GENETIC ANALYSIS OF LOW-TEMPERATURE TOLERANCE IN WINTER WHEAT

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By

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ABSTRACT

Winter wheat has a higher yield over spring sown wheat and has many ecological and agronomic advantages. However, low temperature (LT) stress is one of the major abiotic factors which limit increased crop production. LT-tolerance in winter wheat is a quantitative trait and molecular and genetic evidence suggest that LT-tolerance is governed by a number of genes with complex interactions. In order to identify and characterize the chromosomal regions conferring LT-tolerance, a doubled haploid (DH) mapping population was utilized from two winter wheat parents Norstar and Cappelle-desprez which have an identical *vrn-1* locus but differ in their potential LT-tolerance (Båga *et al.*, 2006a; 2006b). Norstar has an LT_{50} of -21°C and Cappelle-Desprez has an LT_{50} of -13°C (Båga *et al.*, 2006a; 2006b). In this study, molecular markers such as simple sequence repeats (SSRs) and amplified fragment length polymorphisms (AFLPs) were used to construct a linkage map of a Norstar x Cappelle-desprez cross and to identify markers and regions associated with LT-tolerance. A linkage map was assembled with a total genetic length of 2292 cM comprising 443 SSR and 197 AFLP markers. A major QTL (Quantitative trait loci- a region of DNA that is associated with a phenotypic trait) for LT-tolerance was identified on chromosome 5A which has been associated with a cluster of C-repeat binding factors (CBFs). The QTL identified in this study is similar to the *Fr-2* locus in diploid and hexaploid wheat, also associated with LT tolerance. A previously constructed Norstar BAC library (Ratnayaka *et al.*, 2005) was screened to identify putative CBF clones. Mapping of the CBF positive clones to the major QTL identified on chromosome 5A will help in identifying the key CBF gene(s) contributing to the LT-tolerance trait.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
AP2	Apetala2
BAP	6-Benzylaminopurine
CBF	C-repeat binding factor
CO	Constans
COR	Cold-regulated
CRE/CRT	C-repeat
DH	Doubled haploid
DHN	Dehydrin
DRE	Dehydration responsive
DREB	Dehydration responsive binding element
HvCBF	*Hordeum vulgare* C-repeat binding factor
ICE	Inducer of CBF expression
K-value	Kruskal-wallis
LD	Long day
LOD	Logarithmic of odds
LT	Low temperature
LT_{50}	Value at which 50% of the plants die due to LT-stress
PPD	Photoperiod
QTL	Quantitative trait locus
SD	Short day

SSR	Simple sequence repeats
TDZ	Thidazuron
VRN	Vernalization

1.0 INTRODUCTION

Low temperature (LT) affects the growth of many commercial crop species at some stage in their life cycle (Sarhan and Danyluk, 1998). Winter wheat (*Triticum aestivum*) acreage in western Canada increased from less than 2,000 acres in 1972 to 860,000 acres in 1985, with Saskatchewan contributing 64% of this total (Fowler, 1992). In the years 1984-1985, on account of severe winterkill along with stem rust and drought, the production declined to an average of about 83,000 acres in 1989 highlighting the impact of the harsh weather conditions (Fowler, 1992).

Winter wheat has certain advantages over spring wheat. Winter wheat has a better yield and can be seeded into stubble from crops such as mustard, canola and peas thereby reducing the risk of insects, diseases and weeds. The stubble acts as a snow trap insulating wheat seedlings against low temperatures. A direct seeding system leaves the stubble relatively undisturbed, which helps in spring soil moisture reserves and also helps to reduce soil erosion (Fowler DB, www.usask.ca/agriculture/plantsci/winter_cereals/index.php). However, the potential for LT-tolerance in existing winter wheat cultivars is not adequate for the severe Canadian winters.

Due to the limited genetic potential of winter wheat, no cultivars superior to the current land races have been bred. Highly adapted winter hardy semi-dwarf cultivars occupy more than 95% of western Canadian winter wheat acreage, and have led to an increase of more than 50% in yield (Fowler DB, www.usask.ca/agriculture/plantsci/winter_cereals/index.php). However, no

progress has been made to increase the LT-tolerance of winter wheat. Understanding and improving LT-tolerance in winter wheat would also address limitations in crops of economic importance.

Hexaploid (bread) wheat, *Triticum aestivum* (2n=6x=42) can be broadly divided into two groupings based on vernalization requirement: winter wheat or spring wheat. Winter wheat is sown in the autumn and it overwinters through the cold winter months whereas spring wheat is seeded in the spring and is not subjected to continuous LT. Three major groups of genes including vernalization response genes (*Vrn*), photoperiod responsive genes (*Ppd*) and earliness *per se* genes (*Eps*) control the flowering time in wheat and are critical to the wide adaptability in wheat (Bullrich *et al.*, 2002). Vernalization genes induce flowering based on the requirement of long exposure to LT while photoperiod responsive genes regulate flowering time in response to daylength. Earliness *per se* genes influence flowering time independently of environmental responses (Bullrich *et al.*, 2002).

The vernalization genes (*Vrn*) have been mapped on the long arm of chromosome 5A (Galiba *et al.*, 1995), 5D and 5B (Sarma *et al.*, 2000) and are the major genes that determine if a wheat is classified as a winter or spring cultivar. Dominant spring alleles at any of the homoeologous group 5 chromosomes (*Vrn-A1*, *Vrn-B1* or *Vrn-D1*) are insensitive to cold treatment and will initiate flowering irrespective of LT exposure, while the recessive winter alleles (*vrn-A1*, *vrn-B1* or *vrn-D1*) normally require at least six weeks of LT before commencement of floral initiation. Vernalization sensitivity is an important

adaptation as it effectively prevents the initiation and development of delicate floral primordial during the winter thereby delaying the transition from the vegetative phase to the reproductive phase.

The changes incurred in flowering time associated with the photoperiod response are also an important adaptation in both winter and spring wheat. Photoperiod response is determined by a homoeologous series of genes, *Ppd-1*, located on the short arm of the group 2 chromosomes. *Ppd-A1, Ppd-B1* and *Ppd-D1* are located on chromosomes 2A, 2B and 2D respectively (Law *et al.*, 1978).

Earliness *per se* effects are determined by genes that control the intrinsic rate of development of primordial numbers, or the rate of vegetative and floral primordial initiation. Wheat chromosomes 2B, 3A, 4B, 4D, 5A, 6B, 6D and 7B have been implicated to have earliness *per se* genes (Flood and Halloram, 1983; Scarth and Law, 1983; Hoogendoorn, 1985; Miura and Worland, 1994).

Vernalization and photoperiod genes in cereals are controlled through separate pathways but eventually converge to influence the expression of LT-induced genes and activate genes involved in plant development (Fowler and Limin, 2004). LT-tolerance is a complex quantitative trait that is expressed following exposure of plants to low, non-freezing temperatures, a process known as cold acclimation (Fowler and Limin, 2004). Cold acclimation is a cumulative process that is initiated in plants when the temperature is below approximately 10° C (Fowler *et al.*, 1999). When plants are grown at constant temperatures in the acclimation range an inverse relationship between temperature and

acclimation rate exists, wherein the most rapid changes in LT-tolerance occur during the initial stages of acclimation (Fowler *et al.*, 2004). It has been proposed that vernalization and photoperiod genes influence the duration of expression of LT-induced structural genes (Fowler *et al.*, 1996, Mahfoozi *et al.*, 2000). In winter wheat, LT and photoperiod sensitivity play an important role as it allows the plant to maintain a high level of LT-induced gene expression during short day (SD), compared to long day (LD) conditions (Mahfoozi *et al.*, 2000).

Molecular mapping, identification and characterization of LT-tolerance genes can help in breeding or genetic manipulation of wheat to overcome the LT-tolerance limitation found in this crop. Molecular mapping has been used successfully to identify chromosomal regions involved in the LT-tolerance of wheat and other cereals. The identified markers are useful in marker-assisted breeding programs for the incorporation of chromosomal regions tightly linked with the identified marker into breeding material without the need for LT screening. Recent genetic and molecular studies of LT-tolerance have shed light on a multisignal transduction network involved in the response to LT (Yamaguchishinozaki and Shinozaki, 1994). The cloning of transcription factors, namely CBFs (C-Repeat Binding Factors), which play important roles in LT-tolerance responses, as well as the identification of *CBF3* in wheat has highlighted the importance of transcription factors that could up-regulate genes responsible for LT-tolerance (Stokinger *et al.*, 1997; Vagujfalvi *et al.*, 2003; Vagujfalvi *et al.*, 2005; Miller *et al.*, 2005).

1.1 THESIS OBJECTIVES

A common objective in plant breeding is to transfer a specific trait to a desirable genotype having an easily remedied effect. In order to remove the major effects of genes in the *Vrn* region, a winter wheat X winter wheat cross using two parents with the same *Vrn* region but differing in LT-tolerance was utilized (Båga *et al.*, 2006a). This may allow for the determination of the genetic control of LT-tolerance in this population. The overall objective of this study is to use molecular mapping techniques to identify chromosomal regions involved in LT-tolerance, other than the *Vrn* region. The specific objectives of this thesis are as follows:

1. To identify chromosomal regions participating in the LT-tolerance of winter wheat using Simple Sequence Repeats (SSRs) and Amplified Fragment Length Polymorphisms (AFLPs);

2. To identify putative CBF transcription factors in winter wheat; and

3. To optimize a mature embryo culture system for various wheat cultivars which may aid in future biotechnological studies with identified LT-tolerance genes.

2.0 LITERATURE REVIEW

2.1 Ancestry of wheat

Wheat (*Triticum aestivum*) (2n=6x=42) belongs to the grass family Poaceae, which includes major crop plants such as rice (*Oryza sativa*), barley (*Hordeun vulgare*), oat (*Avena sativa*) and maize (*Zea mays*) (Sandhu and Gill, 2002). The grass family Poaceae has numerous tribes and wheat belongs to the tribe *Triticeae* which has more than 15 genera and 300 species (Sandhu and Gill, 2002). It is believed that cereals such as wheat (*Triticum*), sorghum (*Sorghum*), rice (*Oryza*) and maize (*Zea*) diverged from a common ancestor about 50 to 70 million years ago from the angiosperm (flowering plants) lineage which is thought to be about 200 million years ago (Kellog, 1998). Wheat species is a polyploidy with three ploidy levels. Hybridization of wild diploid einkorn wheat, *Triticum urartu* (AA genome), with *Aegilops speltoides* (BB genome), a close relative of goat grass resulted in the formation of *Triticum turgidum* ssp. Dicoccoides (AABB genome) (Sarkar and Stebbins, 1956; Dvorak *et al.*, 1993). Hybridization of *Triticum turgidum* (AABB genome) with diploid Aegilops tauschii (DD genome) led to the formation of hexaploid wheat *Triticum aestivum* (AABBDD genome) (McFadden and Sears 1946). The donor of (BB) genome in wheat is presumed to be an unidentified species probably closely related to *Aegilops speltoides* (Levy and Feldman, 2002).

Hexaploid bread wheat is an allohexaploid. Based on the nature of polyploids they are divided as autopolyploids, which are formed by the doubling of a single species genome, and allopolyploids which consist of two or more sets

of related chromosomes due to interspecific fertilization, followed by chromosomal doubling (Wendel, 2000). The large number of allopolyploid plant species in nature suggests that allopolyploids have an advantage over diploid progenitors and is part of an important speciation process (Liu and Wendel, 2003). Allopolyploid formation is also accompanied by rapid genomic changes in several model plant systems (Wendel 2000). Rapid non-mendelian genomic changes could be a possible mechanism for generating *de novo* genomic variations which will result in phenotypic variations under natural conditions and may be partly responsible for the evolutionary success of allopolyploids over diploids (Song *et al.*, 1995; Soltis and Soltis, 1995). Recently, mutants with differences in flowering time were identified from derivatives of allopolyploids within a few generations, revealing the evolutionary significance of the genomic changes (Schranz and Osborn, 2000). However, the genomic change involved in the course of allopolyploid speciation is not clear (Salina *et al.*, 2004). The genomic changes due to polyploidization involves problems of gene dosage, increased genome size, replication of multiple and different genomes, and ensuring pairing between homologs chromosomes while pairing with homoeologs chromosomes are repressed (Levy and Feldman, 2002).

The variation in genome size among major cereals (~490 mb) per 1C for rice (*Oryza sativa*) to (~16,979 mb) per 1C for *Triticum aestivum* may be due to the presense of mobile dispersed repetitive DNA elements present in the genome (Bowers *et al.*, 2003). Repetitive sequences, consisting of transposon elements, comprise 75-80 % of some gene rich regions in cereal genomes

(Flavell *et al.*, 1977). Cytogenetic studies on *triticeae* genomes have shown that the genomes are organized into relatively gene-rich and gene-poor regions (Sandhu and Gill, 2002). Hexaploid wheat has seven groups of chromosomes from 3 genomes (A, B and D), thus chromosomes from group 1, 1A, 1B and 1D have the same gene order and behave as diploids at meiosis, with wheat chromosome 1A pairing with 1A, and not with either 1B or 1D chromosomes (Gill,1991; Gale, 1997). Although most chromosomes of the three wheat genomes present in hexaploid wheat are collinear, chromosome 4A in wheat has undergone several translocations and inversions (Mickelson-Young *et al.*, 1995).

2.2 Development of hexaploid (bread) wheat

Hexaploid (bread) wheat (*Triticum aestivum*) is one of the world's most important cereal crops and exists as a polyploidy species of diploid, tetraploid and hexaploid (Provan *et al.*, 2004). Studies on nuclear encoded genes, the plastid acetyl CO-A carboxylase (ACC1) and 3-phosphoglycerate kinase (PGK-1), indicate the hybridization of *Triticum urartu* (AA) with an unknown B genome species to have occurred less than 0.5 million years ago and the ancestral diploid A, B and D genomes to have diverged between 2.5 and 4.5 million years ago (Huang *et al.*, 2002). Hybridization of *Triticum turgidum* ssp. *diciccoides* (AABB genome), a tetraploid wheat ancestor, with *Aegilops tauschii* (DD genome), led to the development of *Triticum aestivum* (2n = 6x = 42, AABBDD genome) around 8,000 to 10,000 years ago (Huang *et al.*, 2002; Salamini *et al.*, 2002). It is believed that hexaploid wheat originated in northwestern Iran or northwestern

Turkey as a result of hybridization between tetraploid wheat and diploid wheat *Aegilops tauschii* followed by chromosome doubling (McFaddens and Sears, 1946). Recently, studies using DNA markers such as microsatellites or simple sequence repeats (SSRs) have allowed the detection of polymorphism in populations derived from intraspecific or intervarietal crosses and helped in establishing the allelic diversity of wheat. An analysis of 559 French bread wheat accessions (land races and cultivars from 1800 to 2000) with microsatellite markers has showed that there is a decrease in allelic diversity (Roussel *et al.*, 2005). Studies performed using microsatellite markers to assess changes in allelic diversity in European bread wheat and to compare the level of the distribution of genetic diversity in 480 European wheat varieties as a function of their geographical origin has provided valuable information regarding the diversity in European wheat accessions (Roussel *et al.*, 2005). The study sampled 480 bread wheat varieties from 15 European geographical areas released from 1840 to 2000, and found that the total number of alleles present in wheat varieties were stable until the year 1960 and thereafter decreased considerably (Roussel *et al.*, 2005). The study also found that European accessions were more differentiated as a function of their geographical origin. Western European countries (France, Great Britan, The Netherlands, Belgium) displayed a lower number of alleles than south eastern European countries (Greece, Bulgaria, Romania and Hungary) reflecting the different breeding practices (Roussel *et al.*, 2005). Analysis of the genetic diversity of 55 winter wheat cultivars grown in the UK from 1934 to 1994 has revealed a shift in genetic

diversity probably due to the introgression of novel genes and the use of semi-dwarf wheat germplasm (Donini *et al.*, 2000). Analysis of the genetic variability of 75 Canadian hard red spring wheat cultivars released from 1845 to 2004 using SSR markers has shown that 51 alleles (19%) present in pre-1910 cultivars were not present in cultivars released after the year 1990 (Fu *et al.*, 2005a).

2.3 Spring and winter wheat

In wheat (*Triticum aestivum*), vernalization and photoperiod responses allow the plant to synchronize its growth and reproductive cycle with seasonal changes (Fowler *et al.*, 2004). Flowering is controlled predominantly by vernalization (*Vrn*) and photoperiod responsive genes (*Ppd*). Vernalization genes induce flowering based on the requirement of exposure to LT whereas photoperiod responsive genes regulate flowering time in response to day length. Winter wheat has a vernalization requirement and requires exposure to LT whereas spring wheat lacks this requirement. Winter wheat is sown in the autumn and it overwinters through the cold winter months, whereas spring wheat is seeded in the spring and is not subjected to continuous cold temperatures. Vernalization and photoperiod genes are controlled through separate pathways but eventually converge to influence the expression of LT-induced genes (Fowler *et al.*, 1996; Mahfoozi *et al.*, 2000; Fowler and Limin, 2004).

2.3.1 Vernalization

Vernalization is defined as the acquisition or acceleration of the ability to flower by a chilling treatment (Chouard, 1960) and this process plays an important role in determining the growth habit of wheat (Limin and Fowler, 2002). Studies have established that vernalization causes changes in specific DNA methylation patterns that are not transmitted through meiosis (Finnegan *et al.*, 1998). In Arabidopsis, the vernalization pathway renders Arabidopsis able to flower after long exposure to cold and is mediated by repressing the expression of the *Flowering locus C* (*FLC*) (Michaels and Asmasio, 2000). The homoeologous group 5 chromosomes with genes *Vrn-A1* (formerly *Vrn1*), *Vrn-B1* (formerly *Vrn2* or *Vrn4*) and *Vrn-D1* (formerly *Vrn3*) located on 5A, 5B and 5D respectively are responsible for the vernalization response in wheat (Law *et al.*, 1975; Law and Worland, 1997). The *vrn-1* allele determining winter habit is recessive to the dominant allele *Vrn-1* determining spring habit, and hence, all alleles at the homologous *Vrn-1* locus should be recessive to produce a winter habit plant (Pugsley, 1971). Vernalization gene *Vrn-B4* (*Vrn5*) has been located on the 7B chromosome and additional *Vrn* genes have been identified on homoeologous group 6 chromosomes (Islam-Faridi *et al.*, 1996; Hay and Ellis, 1998). The developmental delay due to the vernalization requirement is an adaptation mechanism of winter habit plants, helping them to accumulate LT-tolerance by remaining in the vegetative phase (Limin and Fowler, 2002). In diploid wheat (*Triticum monococcum*), a positional cloning approach identified the wheat vernalization gene *Vrn1* (*AP-1*) (Yan *et al.*, 2003). The *Vrn1* gene is a member of the MADS-box family of transcription factors. The presence of the

MADS-box in the promoter of *Vrn1* suggests that a trans-acting factor may bind at this site (Yan *et al.*, 2003). Positional cloning of the *Vrn2* gene, a flowering repressor down-regulated by vernalization, has shown that *Vrn2* gene encodes a zinc finger-CCT domain transcription factor and represses flowering by preventing the expression of *Vrn1* (Yan *et al.*, 2003; 2004a). A mutation or complete deletion of the *Vrn2* gene is associated with the recessive spring growth habit in wheat and barley (Yan *et al.*, 2004b). Studies on the *Vrn1* gene from hexaploid spring wheat accessions have shown that there are large deletions within a 4 kb region in the first intron in the *Vrn1* gene (Fu *et al.*, 2005b). The study also revealed a 2.8 kb region within the 4 kb region having high sequence conservation among the different alleles (Fu *et al.*, 2005b). It is hypothesized that the conserved 2.8 kb region may include regulatory elements responsible for vernalization requirement and the deleted region within the intron 1 may include a recognition site for the flowering repressor, expressed from the *Vrn2* gene (Fu *et al.*, 2005b).

Recently, the *TaVRT-1* gene (*Triticum aestivum* vegetative to reproductive transition-1) has been identified and encodes a protein homologous to the MADS-box family of transcription factors which are responsible for flowering repression (Danyluk *et al.*, 2003). The expression of *TaVRT-1* is associated with the vernalization response and the transition from the vegetative phase to the reproductive phase. In addition, it is negatively associated with the accumulation of COR (cold-regulated) genes (Danyluk *et al.*, 2003). This gene is constitutively expressed in spring habit genotypes but induced by LT only in those species that

require vernalization. The expression of *TaVRT-1* is also regulated by photoperiod, suggesting that vernalization and photoperiod may regulate this gene through separate pathways (Danyluk *et al.*, 2003). Characterization of the *TaVRT-2*, also encoding a MADS-box transcription factor, has revealed that *TaVRT-2* interacts with other vernalization genes (*TaVRT-1/Vrn1* and *Vrn2*) in wheat (Kane *et al.*, 2005).

2.3.2 Photoperiod

In cereals such as wheat and barley, photoperiod sensitivity allows plants to maintain LT-tolerance for a longer period of time under short-day (SD) conditions compared with long-day (LD) conditions (Mahfoozi *et al.*, 2001). The delay incurred for the transition from the vegetative to the reproductive phase ensures that LT-tolerance is sustained. Once this switch is made, there are minimal increases in LT-tolerance (Fowler *et al.*, 1996; Mahfoozi *et al.*, 2001). The genes involved in photoperiod response in wheat have been mapped to the group 2 chromosome (Law *et al.*, 1978; Scarth and Law, 1983). Although homologous group 1 chromosomes have been implicated in vernalization and photoperiod responses in wheat, the major photoperiod genes *Ppd-A1* (formerly *Ppd3*), *Ppd-B1* (formerly *Ppd2*) and *Ppd-D1* (formerly *Ppd1*) are located on the short arms of wheat chromosomes 2A, 2B and 2D respectively (Law *et al.*, 1978; Scarth and Law, 1983).

In Arabidopsis, the *CONSTANS* (*CO*) gene plays an important role in the photoperiod response (Putterill *et al.*, 1995). In Arabidopsis, *CO* is controlled by a

circadian rhythm and activated by LD. *CO* promotes the transcription of two important genes involved in floral transition, *Flowering Locus T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Samach *et al.*, 2000; Kardailsky *et al.*, 1999). Expression of the *FT* and *SOC1* genes induces the expression of downstream meristem identity genes which signals the apical meristem to switch from the vegetative phase to the reproductive phase (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). *CO*-like genes have been identified in various other plants such as *Brassica napus* (*BnCOa1*) (Robert *et al.*, 1998). In cereals, such as rice (Oryza sativa), the *Heading date 1* (*Hd1*) gene is homologous to *CO* (Yano *et al.*, 2000). Rice is a SD plant and *Hd1* promotes heading under SD conditions but suppresses heading under LD (Yano *et al.*, 2000). The *Hd1* gene has been isolated by positional cloning and the amino acid sequence contains two zinc finger domains and a nuclear localization signal similar to the *CO* gene from Arabidopsis (Yano *et al.*, 2000). In wheat, the *TaHd1* gene has been isolated which is the homolog of *Hd1* in rice (Nemoto *et al.*, 2003). The *TaHd1* gene complemented the loss of *Hd1* function in rice, demonstrating that *Hd1* and *CO* genes from LD plants can replace their homologs from SD plants (Nemoto *et al.*, 2003). The photoperiod response is important with regard to flowering time and represents an important adaptation in winter and spring wheats (Bonjean and Angus, 2000). Due to photoperiod sensitivity, spring wheat cannot be grown as an overwintering crop in tropical areas because at low latitude, the day-length requirement would never be satisfied (Bonjean and Angus, 2001).

2.4 Low-temperature (LT) stress

When plants are exposed to temperatures below 0°C, ice starts to form in the intercellular spaces of plant tissue (Thomashow, 1998). Since the chemical potential of ice is less than that of liquid water, when intercellular ice formation takes place there is a decrease in water potential outside the cell and unfrozen water from inside the cell is moved to the intercellular spaces (apoplast) down the chemical potential gradient (Thomashow, 1998). The intercellular volume of the cell decreases when extracellular ice forms and often results in expansion-induced lysis (Wolfe *et al.*, 1985). Thus, freezing injury results from the severe cellular dehydration that occurs (Levitt, 1980). Plants are subjected to multiple stresses when water freezes in the apoplast: the ion and solute concentrations are increased; proteins, lipids and other maromolecules are affected; and plasma membrane integrity is compromised, leading to an onset of expansion-induced lysis and loss of osmotic responsiveness (Levitt, 1980; Steponkus, 1984).

However, LT-tolerant winter cereals can survive freezing events as a result of a process referred to as cold acclimation (Guy, 1990; Huner *et al.* 1993; Thomashow, 1998; Xin and Browse, 20000). The exposure of these plants to low, non-freezing temperatures initiates a series of various genetic and biochemical events by which the plant acclimates to the lower temperature and improves freezing tolerance (Guy, 1990; Thomashow, 1998; Xin and Browse, 2000). The freezing tolerance trait is used to estimate the threshold potential of a plant to withstand freezing. However, the acquisition of freezing tolerance is

dependent upon many factors and the genetic potential of the plant may never be realized. Freezing tolerance is typically measured experimentally by assessing re-growth after freezing and determining a LT_{50} value, which is defined as the temperature where 50% of the plants are killed by LT-stress (Levitt,1980).

2.5 Biochemical changes associated with LT-tolerance

Plants have evolved various biochemical and genetic mechanisms that are induced upon exposure to LT (Guy, 1990). These mechanisms assist in preventing, reversing or reducing the damage caused by the accumulation of intercellular ice and in preventing cellular molecules from precipitating and freeze-induced denaturation of proteins (Thomashow, 1998).

Alterations of lipid composition in the cellular membrane play an important role in LT-tolerance in plants (Nishida and Murata, 1996). Plasma membranes with a high proportion of unsaturated lipids have been shown to be more tolerant to expansion-induced lysis and resistant to freeze-induced membrane lesions (Uemura and Steponkus, 1999).

Plants also accumulate cryoprotective proteins which protect membranes from freeze-induced damage by reducing the membrane permeability during freezing and by increasing membrane expandability during thawing (Hincha *et al.*, 1990). These are referred to as dehydrins and late embryogenesis abundant (LEA) proteins, which are induced, by a variety of environmental stresses (Wisniewski *et al.*, 1999). In evergreen and deciduous peach, studies have demonstrated that dehydrin protein expression is correlated to the qualitative and

quantitative differences in LT-tolerance (Wisniewski *et al.*, 1999). LT exposure can also lead to the accumulation of antifreeze proteins. These proteins protect the plants by adsorbing to the surface of ice crystals by Vander Waals interaction or hydrogen bond interaction, and inhibit the recrystallization and growth of intercellular ice (Knight and Dunman, 1986). In plants, antifreeze proteins are similar to pathogenesis-related proteins that are secreted in response to pathogens (Hon *et al.*, 1995). Immunolocalization experiments have shown that the antifreeze proteins accumulate in the mesophyll cell walls, in the secondary cell walls of xylem vessels, and in epidermal cell walls (Griffith *et al.*, 1997).

Compatible osmolytes, also known as osmoprotectants, are compounds that are highly soluble, have no net charge at physiological pH and are non-toxic at high concentrations (McNeil *et al.*, 1999). Osmoprotectants play an important role in LT-tolerance by stabilizing the proteins and membranes and by raising the osmotic pressure in the cytoplasm (McNeil *et al.*, 1999). LT exposure leads to the accumulation of low-molecular-weight organic solutes such as proline, glycine betaine and sugars (Olien and Clark, 1993; Dorffling *et al.*, 1997; Crowe *et al.*, 1998; Sakamoto and Murata, 2001).

2.6 LT-induced genes

Exposure to LT leads to the expression of a number of genes (Thomashow, 1994). LT-induced genes encode proteins such as antifreeze proteins, molecular chaperones and enzymes involved in lipid biosynthesis,

respiration, antioxidants and metabolism of carbohydrates (Guy, 1990), many of which have been summarized in the preceding section.

The cold-responsive genes are grouped based on the amino acid sequence of the encoded polypeptides (Thomashow, 1994). Most of the polypeptides encoded by the cold-responsive genes are extremely hydrophilic (Thomashow, 1994). In Arabidopsis, some of the cold-responsive genes include *COR* genes (cold-regulated), *LTI* (low temperature-induced), *RD* (responsive to desiccation), *KIN* (cold-inducible) and *ERD* (early dehydration-inducible) (Thomashow, 1994).

In Arabidopsis, some of the *COR* regulon consists of *cor6.6*, *cor15*, *cor47* and *cor78*, (Thomashow, 1994; 1998). The *cor15* gene is the best characterized and is expressed in response to LT, drought and abscisic acid (ABA). This gene encodes a 15 kD polypeptide that is targeted to the chloroplast where it is processed to the 9.4 kD polypeptide COR15am and is thought to stabilize thylakoid membranes against freeze-induced injury (Thomashow, 1994, 1998). The *cor47* gene encodes a hydrophilic 'boiling soluble' polypeptide which belongs to the LEAII protein family, also known as dehydrins (Welin *et al.*, 1994, 1995). LEA proteins are usually synthesized during embryo maturation but are also induced in response to LT, high salinity and drought stress (Ingram and Burtels, 1996). All LEAII protein members have K-segments (lysine-rich sequences) and are likely to form amphipathic α-helices (Close, 1997). Dehydrin genes (*Dhn*1, *Dhn*2, and *Dhn9*) have been mapped on chromosome 5H in barley (Choi *et al.*, 2000). In Arabidopsis, transcriptome profiling has identified multiple

regulatory pathways that are activated during LT exposure (Fowler and Thomashow, 2002). In response to LT treatment, 218 genes were up-regulated and 88 genes were down-regulated (Fowler and Thomashow, 2002). Analysis of cold-responsiveness of 22,043 genes in Arabidopsis as a function of exposure time for 14 days at 4°C has helped in identifying the metabolic pathways and physiological process that are predominantly involved in the plant cold-acclimation process (Hannah *et al.*, 2005).

In barley, the *cor14b* gene is expressed when plants are exposed to LT and is regulated by light (Crosatti *et al.*, 1995, 1996). The 14 kDa COR14 protein accumulates in the chloroplast stroma (Crosatti *et al.*, 1995). The threshold temperature for induction of *cor14b* is lower in less-hardy than in hardy cultivars (Crosatti *et al.*, 1995, 1996) and the expression level is greater in winter cultivars as compared to spring cultivars (Giorni *et al.*, 1999). In diploid wheat *(Triticum monococcum)*, the barley *cor14b* homolog has been mapped to the long arm of chromosome $2A^m$ (Vagujfalvi *et al.*, 2000) that is regulated by a locus(i) located on chromosome 5A of wheat (Vagujfalvi *et al.*, 2000).

In wheat, the *wcs120* gene encodes a member of the LEAII group of polypeptides (Houde *et al.*, 1992). LT regulates the expression of *wcs120* which encodes the 50 kDa WCS120 protein (Houde *et al.*, 1995). WCS120 is highly hydrophilic, remains soluble upon boiling and is composed of a K-segment that is repeated six times. The WCS120 protein is expressed more abundantly in the crown tissue and leaves compared to roots and may be the reason for differential freezing tolerance in tissues (Houde *et al.*, 1992). Homologs of *wcs120* are

present in Gramineae species such as rice and corn which are chilling-sensitive but the *wcs120* gene is not induced by LT, probably due to the absence of *cis*/*trans*-acting factors (Danyluk *et al.*, 1994). In winter wheat, the accumulation of both *wcs120* mRNA and protein are closely correlated with the ability to develop freezing tolerance (Limin *et al.*, 1995). It is also interesting to note that the *wcs120* gene copy number and gene organization are identical in both hardy and less-hardy wheat cultivars (Limin *et al.*, 1995). In wheat, other low-temperature regulated genes include the *wcs200*, *wcs66*, *wcs19*, *wcor14*, *wcor15* and *wcor410* (Hughes and Dunn, 1996). The *wcs19* gene is regulated by LT but also requires light for maximum induction (Chauvin *et al.*, 1993). Overexpression experiments have shown that wheat *wcs19* increases the LT-tolerance in Arabidopsis (Dong *et al.*, 2002). The wheat ortholog of barley *cor14b*, *wcor14* as well as *wcor15* are also induced by LT and light (Tsvetanov *et al.*, 2000; Takumi *et al.*, 2003). Wheat and related wild genomes possess multiple copies of *wcor15* homologues (Takumi *et al.*, 2003). The *wcor15* gene encodes a chloroplast-targeted protein and has a conserved CRT/DRE (C-repeat/ Dehydration responsive) like sequence motif in their promoter sequence (Takumi *et al.*, 2003). In addition, LT-tolerant winter cultivars accumulate a greater amount of *wcor15* transcripts than LT-sensitive spring cultivars (Takumi *et al.*, 2003; Kobayashi *et al.*, 2004). The *wcor410* gene is LT responsive and encodes an acidic dehydrin protein which accumulates in the vicinity of the plasma membrane of cells where freeze-induced dehydration occurs. It is thought to prevent the destabilization of the plasma membrane that occurs due to

freeze-induced dehydrative conditions (Danyluk *et al.*, 1998). The expression of WCOR410 is also closely correlated with the capacity of the cultivar to develop freezing tolerance (Danyluk *et al.*, 1998). In hexaploid wheat, the *wcor410* gene belongs to a family of three homologous members *wcor410*, *wcor410b* and *wcor410c* which have been mapped to the homologous group 6 chromosomes (Danyluk *et al.*, 1998).

In wheat, the WLT10 protein is expressed during low-temperature and belongs to a cereal-specific COR protein family (Ohno *et al.*, 2001). A LT-tolerant winter wheat cultivar accumulates *wlt10* mRNA more rapidly than the spring cultivar (Ohno *et al.*, 2001). The expression of *wlt10* mRNA is temporal but its maximum expression correlates with the maximum level of freezing tolerance attained (Ohno *et al.*, 2001). The *wlt10* homologues from barley have been mapped to the homologous group 2 chromosomes (Ohno *et al.*, 2001). Recently, the *wpi6* gene, which encodes a plasma membrane protein WPI6, has been identified in wheat and is induced following LT exposure (Imai *et al.*, 2005). The WPI6 protein belongs to the BLT101 protein family from wheat (Imai *et al.*, 2005). In barley, the *blt101* gene is also expressed following LT exposure and accumulates in the crown tissues (Goddard *et al.*, 1993).

Transcriptome analysis of winter and spring wheats over the course of 36 days of LT exposure has revealed differences in the regulation of LT-induced genes between the two cultivars (Gulick *et al.*, 2005). Comparison of the less-hardy spring cultivar with the hardy winter cultivar demonstrated that a total of 87 genes were differentially regulated (Gulick *et al.*, 2005). The transcript levels of

309 genes were altered (increased or decreased) in the winter cultivar while only 166 gene transcript levels were altered in the spring cultivar (Gulick *et al.*, 2005).

2.6.1 Regulation of LT-induced genes

Analyses of the promoters of the genes induced by LT and drought have identified ABA-dependent and ABA-independent mechanisms of regulation for LT-induced genes. Two major *cis*-acting elements; the ABA-responsive element (ABRE) and the dehydration responsive element/C-repeat (CRE/CRT), function in ABA-dependent and ABA-independent gene expression, respectively, during LT and osmotic stress responses. The ABRE has a conserved G-box sequence (CACGTGGC) while the dehydration responsive element (DRE) has a 9 bp conserved sequence (TACCGACAT) (Menkens *et al.*, 1995; Yamaguchishinozaki and Shinozaki, 1994). *Apetela2* (AP2) domain transcriptional activators, known as CBF (C-repeat Binding Factor) or DREB1 (DRE binding) proteins bind to the CRT/DRE element to activate transcription of LT-induced genes (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Liu *et al.*, 1998; Shinwari *et al.*, 1998). In Arabidopsis, members of the CBF/DREB1 family; *CBF1*, *CBF2* and *CBF3* (or *DREB1b*, *DREB1c* and *DREB1a* respectively) are expressed within 15 minutes of LT exposure and induce downstream expression of the *cor* regulon (Gilmour *et al.*, 1998; Liu *et al.*, 1998; Jagglo-Ottosen *et al.*, 1998). Recently, the gene *inducer of CBF expression 1* (*ICE1*) was identified (Chinnusamy *et al.*, 2003). *ICE1* encodes an MYC-like bHLH transcriptional activator that binds specifically to the MYC recognition sequence in the *CBF3* promoter (Chinnusamy *et al.*,

2003). It has also been shown that *ICE1* is constitutively expressed and its overexpression induces the expression of the *CBF3* and improves LT-tolerance (Chinnusamy *et al.*, 2003). The *CBF* LT pathway is conserved and components of this pathway are present in LT tolerant cereals such as wheat, barley and rye, as well as in LT sensitive plants such as tomato (Jaglo *et al.*, 2001). Studies have shown that while tomato has a *CBF* LT pathway, it differs from that of Arabidopsis and appears to be less diverse in function (Zhang *et al.*, 2004). Recently, a non-CBF transcription factor, ZAT12, has been identified which functions independently from the CBF pathway (Vogel *et al.*, 2005). In Arabidopsis, the constitutive expression of the ZAT12 regulon causes a small increase in LT-tolerance while down-regulating the expression of *CBF* genes and thus may be a negative regulator of the CBF pathway (Vogel *et al.*, 2005).

In wheat, CBF/DREB homologues such as *TaDREB1* have been identified (Shen *et al.*, 2003). The *TaDREB1* gene is induced by LT, drought and salinity. The expressed TaDREB1 protein has a conserved ethylene responsive binding domain (EREBP/AP2) similar to the DREB family members of Arabidopsis (Shen *et al.*, 2003). A LT-regulated transcriptional activator (*CBF3*) has been identified in wheat which is linked to the LT-tolerance locus *Fr-A2* on chromosome 5A (Vagujfalvi *et al.*, 2003). In *Triticum monococcum* it has been shown that expression of several genes at the *Fr-A2* locus are linked to LT-tolerance and a cluster of 11 *CBF* transcription factors are located at this locus on chromosome 5A (Miller *et al.*, 2005). In barley, the CBF/DREB1 homologues *HvCBF3*, *HvCBF4* and *HvCBF8* have been mapped on the 5H-L chromosome in two

tandem clusters (Choi *et al.*, 2002; Francia *et al.*, 2004; Skinner *et al.*, 2005). The linkage map positions of the ICE1 homolog, ZAT12 homolog and 17 CBF homologs have also been identified in barley (Skinner *et al.*, 2005).

2.7 Genetic mapping

Linkage map construction requires 1) a mapping population, derived from crosses between homozygous parents or heterozygous parents in the case of plants intolerant to inbreeding, 2) identification of polymorphisms in the parents using different marker techniques and 3) linkage analysis of the markers. Usually, genetic maps are constructed based on 50 to100 individuals, but for high resolution mapping additional individuals are required (Paterson, 1996). It is essential that the parents selected for developing the mapping population differ in the trait of interest.

2.7.1 Mapping populations used for genetic analyses

2.7.1.1 Doubled Haploid (DH) lines

Doubled haploid plants are completely homozygous and contain two identical sets of chromosomes in each cell. Doubled haploids are usually produced *in vitro* by anther culture or by interspecific hybridization (Choo, 1981). The first doubled haploids of wheat were produced by anther culture (Ouyang *et al.*, 1973). Chromosome doubling is achieved by treating the haploid plants with colchicine, which prevents the formation of spindle apparatus during mitosis leading to the formation of doubled haploid cells. In wheat, haploid embryos can

also be generated by using pollen from the maize species (Zenkteller and Nitzsche, 1984). During cell division of the embryo, the wild species chromosome is eliminated leading to a haploid chromosomal set derived from the egg cell which can be doubled using colchicine treatment. In the doubled haploid lines, homozygosity is achieved in a single step and the population obtained is a stable permanent population that is genotypically homozygous or true breeding (McCouch and Doerge, 1995).

2.7.1.2 F2 populations

Selfing the F1 hybrid, which is the result of a cross between two parents, results in an F2 population. The F2 plants undergo only one cycle of meiosis and hence contain unique recombination events (Paterson, 1996). The F2 population also consists of all possible combinations of parental alleles and has more information than a backcross population, which helps in determining the order of genetic markers on the chromosome compared to a backcross population (Paterson, 1996). The F2 population thus helps in the estimation of dominance components of the quantitative trait loci (QTL) which is not possible with the backcross population (Slate, 2005). However, F3 plants derived from selfing F2 plants are not genetically identical and cannot be preserved, which is considered as one of the disadvantages of using an F2 population (Meksen and Khal, 2005).

2.7.1.3 Recombinant inbred lines (RILs)

Recombinant inbred lines are developed by recurrent selfing of the F2 plants. This method is also known as the single-seed decent method (Paterson, 1996). In this method, one seed from each line is the source for the next generation and within six generations, the plants are completely homozygous. Because of the homozygous nature of the RILs, further genotyping of the lines are not necessary and the lines can be permanently propagated and used indefinitely (Burr and Burr, 1991). Since recombinant inbred lines undergo several rounds of meiosis to attain homozygosity, they exhibit a higher degree of recombination and generate a higher resolution genetic map compared to the F2 population (Burr and Burr, 1991). In some cereals such as rice, *in vitro* culture of doubled haploids is not possible as certain rice genotypes are not responsive to tissue culture. Thus, RILs are the method of choice for attaining homozygosity in the population (McCouch and Doerge, 1995).

2.7.2 Marker techniques for identification of polymorphisms

A molecular marker is defined as a region of DNA showing sequence polymorphism in different individuals within a species (Liu, 1998). Polymorphism is defined as a detectable heritable variation at a particular locus (Liu, 1998). Other types of genetic markers include biochemical markers, which identify allelic variants of enzymes called isozymes, and morphological markers which are related to phenotypic trait or characters (Patterson, 1996). DNA markers can be broadly classified as hybridization-based and polymerase chain reaction (PCR) based (Liu, 1998). Based on the ability of DNA markers to

distinguish homozygotes and heterozygotes, the markers can be described as co-dominant or dominant (Liu, 1998).

2.7.2.1 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism analysis is based on restriction digestion and hybridization. In RFLP, restriction endonucleases are used to make sequence-specific cuts in the DNA. The restriction digestion of DNA is subjected to electrophoresis to separate the DNA fragments based on their size. The DNA fragments are denatured and the single-strand DNA fragments are transferred to a nitrocellulose membrane. A radioactively labeled probe is used to probe the blot containing the transferred DNA fragments. An X-ray film is exposed to the Southern blot to visualize the DNA-probe hybridization (Botstein *et al.*, 1980). RFLPs can distinguish between homozygous and heterozygous genotypes and hence can be scored as co-dominant markers (Liu, 1998). However, if the DNA probe hybridizes to repeated sequences at multiple locations, allelic and non-allelic variations cannot be identified (Liu, 1998). The RFLP technique requires 50 to 200 micrograms of DNA and is considered as a major disadvantage compared to PCR based markers (Paterson, 1996). RFLP procedure is also expensive, laborious and time consuming and exhibits limited polymorphism (Tanksley *et al.*, 1989).

2.7.2.2 Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA marker system uses an identical pair of short primers 8 nt to 10 nt in length at low annealing temperature to randomly amplify segments of nuclear DNA in a PCR based reaction (Williams *et al.*, 1990). RAPD markers are scored as dominant markers and can amplify multiple loci using a single primer. The amplified products obtained by using an RAPD arbitrary primer is a function of genome length, maximum fragment length that can be amplified and the number of nucleotides present in the primer (Liu, 1998). RAPD primers are commercially available and can be used as a marker system for many plants and animal species as designing RAPD primers does not require specific sequence information. However, RAPD marker results are not always reproducible, which is a major disadvantage.

2.7.2.3 Simple Sequence Repeats (SSRs)

SSRs, also called microsatellites, consist of repeated sequence of DNA ranging in size from 1 to 6 bp (Tautz, 1989). The difference in number of repeat units is believed to be caused by polymerase slippage during DNA replication (Tautz, 1989). SSR markers are PCR based markers and the variation in the SSRs can be detected using primers flanking the repeats. SSR markers are scored as co-dominant markers, have a high level of allelic diversity, require small amounts of DNA, and the results obtained are reproducible (McCouch *et al.*, 1997). However, the production of SSR primers and the requirement of polyacrylamide gel electrophoresis to detect polymorphism by SSR markers is time-consuming and laborious (Powell *et al.*, 1996). SSR markers are usually

designed by constructing and screening a genomic library with probes complementary to SSR sequences (Roder *et al.*, 1995; Taramino and Tingay, 1996) or by screening for SSR sequences in the database (Cho *et al.*, 2000). For generating SSR markers from a library, genomic DNA is isolated, digested with restriction enzymes and subjected to electrophoresis to select fragments in the size range of 300 bp to 1000 bp. The selected fragments are ligated to a vector and hybridized with probes comprising several repeat sequences. The positive clones are sequenced and primers are designed based on the flanking region (Roder *et al.*, 1995; Taramino and Tingay, 1996). Database searches containing SSR sequences in databases such as EMBL and Genbank can also be used to develop SSR markers (Cho *et al.*, 2000). However, studies have shown that SSR markers designed by screening databases exhibit a lower level of polymorphism than SSR markers derived from genomic libraries (Cho *et al.*, 2000). Alternative methods to design SSR markers have been proposed that circumvent the construction of a genomic library (Cifarelli *et al.*, 1995). Random amplified hybridization microsatellite (RAHM) method has been developed to isolate a new class of SSR marker containing DNA clones (Cifarelli *et al.*, 1995). This procedure relies on the fact that RAPD fragments contain SSRs more frequently than random genomic clones (Cifarelli *et al.*, 1995). In this procedure, a PCR reaction is performed with SSR anchored random primers or with RAPD primers. The PCR products obtained are electrophoresed on an agarose gel followed by Southern hybridization of the PCR products with SSR probes which will identify the positive bands (Cifarelli *et al.*, 1995). In wheat, SSR marker

sequences has been published by several groups (Roder *et al.*, 1995; Devos *et al.*, 1995; Korzun *et al.*, 1997; Roder *et al.*, 1998; Pestsova *et al.*, 2000; Song *et al.*, 2002; Gao *et al.*, 2004; Nicot *et al.*, 2004; Song *et al.*, 2005).

2.7.2.4 Amplified fragment length polymorphism (AFLP)

AFLP marker system is a PCR-based fingerprinting technique that reveals high level of polymorphism at multiple loci (Vos *et al.*, 1995). In AFLP analysis, the genomic DNA is digested with a 6 bp restriction enzyme (*Eco*RI, a 'rare cutter') and a 4 bp restriction enzyme (*Mse*I, a 'frequent cutter'). Adaptors of known sequences are ligated to the restriction site generated by the two restriction enzymes. Primers are designed based on the adaptor sequences to amplify different subsets of the restriction-digested and adaptor sequence ligated fragments. Changing the selective extension bases of the primers will result in the amplification of a different subset of the restricted digested and adaptor-ligated fragments. The selective extension bases of the primer can be changed, thus amplifying different subsets of the fragments. The PCR products are separated on a denaturing polyacrylamide gel (Vos *et al.*, 1995). The AFLP marker is scored as a dominant marker (Liu 1998). The major advantage of the AFLP marker system is the high number of polymorphism revealed at multiple loci and high reproducibility of the results (Vos *et al.*, 1995).

2.7.3 Linkage analysis of markers

Commonly used software programs in the linkage analysis of markers include Mapmaker (Lander *et al.*, 1990), Joinmap (Stam, 1993) and Mapmanager QTX (Manly *et al.*, 2001). The linkage between the markers is calculated using odds ratio (the ratio of linkage versus no linkage) which is also referred to as Logarithm of Odds (LOD) value or LOD score (Risch, 1992). A cutoff value of 1000 translates to a 3.0 LOD score between the two markers and indicates that linkage between the markers is 1000 times more likely than no linkage (Hartwell *et al.*, 2004). Markers are grouped into different linkage groups based on the recombination fraction between the markers (Liu, 1998). Markers within linkage groups are ordered to determine their relative position on the genetic map (Liu, 1998). To convert recombination fractions into centimorgans (cM), mapping functions such as Kosambi mapping function, which assumes recombination events influence the occurrence of adjacent recombination events and the Haldane mapping function (Haldane, 1919), which assumes no interference between crossover events are required are used (Liu, 1998). Mapping functions compensate for inaccuracies in relating recombination frequencies to physical distances and make corrections for recombination frequency values of widely separated genes that lie close together (Hartwell *et al.*, 2004). However, corrections for large distances are not accurate as the mapping function is based on assumptions, such as no interference (Hartwell *et al.*, 2004). Making use of smaller marker intervals, and by locating widely separated genes through linkage to common intermediaries, are alternative options to create an accurate map (Hartwell *et al.*, 2004). A linkage map is deemed complete when the number of

linkage groups is equal to the number of gametic chromosomes in the organisms, and when the genetic markers that are mapped show genetic linkage to existing markers (Paterson, 1996).

2.8 Physical mapping

Physical maps reveal the actual physical distance between loci, measured in physical units of base pairs, and is based on determining the correct order of overlapping genomic DNA fragments which are cloned into a vector such as a yeast artificial chromosome (YAC) or a bacterial artificial chromosome (BAC). While the distance between the markers in a genetic map is based on recombination units measured in centimorgan, the physical distance is measured in base pairs between the two genetic markers. In higher plants, the physical quantity of DNA corresponding to 1 centimorgan distance in a genetic map varies from 280 kb in Arabidopsis to more than 7000 kb in barley and can vary substantially at different locations within the genome (Paterson, 1996).

2.8.1 Large insert libraries

A large insert DNA library is important for the development of a physical map and many cloning systems have been developed for constructing physical maps of large genomic regions. A cosmid vector developed by Collins and Hohn in 1978, which could maintain up to 50 kb DNA was used for generating the physical map of C. elegans (Coulson *et al.*, 1986). Development of yeast artificial chromosomes (YACs) improved the prospects of considering large scale

projects such as the assembly of contigs representing entire chromosomes of higher eukaryotes (Burke *et al.*, 1987). Yeast artificial chromosome vectors can maintain DNA inserts of 300 kb to 1000 kb (Burke *et al.*, 1987). A YAC library containing more than 10 equivalents of the Arabidopsis genome was constructed with an average insert size of 150 kb (Grill and Somerville, 1991). Using this YAC library, genes conferring abscisic acid insensitivity abi-1 and abi-2, were fine mapped and the corresponding YAC clones isolated (Grill and Somerville, 1991). A YAC library was constructed containing 79,000 clones from the maize genome with an average insert size of 145 kb (Edwards, 1992). A physical map of rice has also been constructed by using a YAC library (Saji *et al.*, 2001). A total of 297 YAC contigs and 142 YAC islands contributed to a physical length of 270 mb which is approximately 63% of the entire rice genome (430 Mb) (Saji *et al.*, 2001). In spite of the advantages of being able to clone a large DNA fragment, YAC inserts have the inherent problem of instability in clones carrying tandem repeated sequences and are often found to be chimeric (Schmidt *et al.*, 1994).

The introduction of the bacterial artificial chromosome (BAC) allowed cloning of a 100 kb to 300 kb DNA fragment in *E. coli* using an F-factor based vector (Shizuya *et al.*, 1992). The F-factor replicon is propagated and maintained in a low copy number and hence has a lower risk of recombination between DNA fragments carried by the plasmid (Shizuya *et al.*, 1992). It is also easy to prepare BAC DNA free of bacterial DNA compared to the isolation of YACs free of yeast genomic DNA. The transformation efficiency of BAC DNA in *E. coli* is also higher compared to the YAC system. In wheat, BAC libraries have been constructed

from diploid, tetraploid and hexaploid wheat (Lijavetzky *et al.*, 1999; Moullet *et al.*, 1999; Cenci *et al.*, 2003; Liu *et al.*, 2000; Nilmalgoda *et al.*, 2003). A hexaploid winter wheat BAC library with an average insert size of 75 kb and genome coverage of 5.5X the haploid genome equivalent has been constructed to isolate genetic loci associated with LT-tolerance (Ratnayaka *et al.*, 2005). In shot-gun sequencing, BACs are a better starting template than YACs for construction of physical maps. Recently, bacteriophage P1-derived artificial chromosomes (PACs) (Ioannou *et al.*, 1994) have also been used to construct physical maps. The PAC system combines the features of the bacteriophage P1 and the F-based BAC cloning system (Ioannou *et al.*, 1994).

2.8.2 Strategies

Two strategies have been successfully employed to sequence complex genomes; a clone-by-clone whole genome sequencing method (International Human Genome Sequencing Consortium, 2001) and whole genome shot-gun sequencing method (Venter *et al.*, 2001). The whole genome shot-gun method does not require a clone-based physical map; instead the genome is fragmented into pieces that are cloned into suitable vectors. The fragments are sub-cloned and the sequences are read from both insert ends. Computational methods using base-calling programs such as Phred are then used to assemble the sequences (Ewing *et al.*, 1998). In the clone-by-clone shot-gun sequencing method, overlapping clone maps named contigs are assembled using data from unique restriction sites and other sequence-based elements in the individual

clones that can be either cloned into YACs or BACs. After the assembly of the contig map, a minimal tiling path which represents minimally overlapping clones are selected for shotgun sequencing. The clones are sub-cloned by random fragmentation and picked at random until a sufficient amount of redundant sequence data is obtained that can be computationally assembled on the basis of sequence overlaps. In Arabidopsis, which has a genome size of ~125 Mb, a clone-by-clone approach was used to construct a physical map (The Arabidopsis Genome Initiative, 2000). In rice, a clone-by-clone strategy was used by the International rice genome sequencing project to sequence the 430 Mbp of rice subspecies *japonica*. Physical maps of 3 chromosomes have been published out of the 12 rice chromosomes (Feng *et al.*, 2002; Sasaki *et al.*, 2002; Rice chromosome 10 sequencing consortium 2003). The whole genome shotgun approach was also used by two rice genome sequencing projects to produce the draft sequences of the rice genome (Yu *et al.*, 2002; Goff *et al.*, 2002). The whole genome shotgun sequence method and the clone-by-clone method can be combined for a hybrid shot-gun sequencing strategy (Myers *et al.*, 2000). In this approach, data sets with enhanced sequence coverage are obtained by combining the whole genome shot-gun sequence information with the corresponding data generated by the clone-by-clone approach, and have been successfully used in the whole genome assembly of Drosophila (Myers *et al.*, 2000).

One of the main problems encountered in constructing a whole genome physical map from plants, especially cereals, is the presense of duplications,

rearrangements, deletions and the high degree of repetitive elements (Arumuganathan and Earle, 1991). In order to circumvent this problem chromosome addition and radiation hybrid lines have been produced in maize for physical mapping (Kynast *et al.*, 2004). The radiation hybrid lines are produced by inducing random chromosomal breaks by lethal radiation of the donor cells and are fused with a recipient cell line from a different species thus allowing the genomic region to be analyzed in a different genetic background thus eliminating the problems caused by repetitive and duplicated sequences (Barret, 1992).

3.0 CHROMOSOMAL REGIONS PARTICIPATING IN LOW TEMPRATURE TOLERANCE

3.1 Introduction

One of the major factors affecting the cultivation of winter wheat is LT. Although breeding winter wheat using the genetic variation in the existing wheat gene pool has provided good results, a better understanding of the genetic factors that contribute to the LT-tolerance potential may allow the breeding of more winter-hardy cereals. Winter wheat also has a higher yield than spring wheat and by breeding more LT-tolerant winter wheat, the improved varieties can be grown over a larger area and this, in turn, will help in boosting the total production of wheat. LT tolerance in wheat is a quantitative trait and is assumed to involve many genes with complex interactions each of which contribute a small effect towards the alleviation of LT-stress (Sutka, 1994). Vernalization and photoperiod sensitivity play a key role in the expression of LT-induced genes (Fowler, 1996). Studies have shown that LT-induced genes are expressed for a longer duration when plants are in the vegetative stage and once the transition from the vegetative to the reproductive stage is completed the LT-induced genes are down-regulated (Fowler, 1996). The LT-induced genes are also up-regulated in wheat and barley under short-day compared with long-day conditions, indicating that photoperiod requirement influences the expression and the duration of expression of LT-induced genes (Mahfoozi *et al.*, 2000).

Cytogenetic studies in LT-tolerance in cereals such as wheat, rye and barley have implicated the group 5 chromosomes for contributing towards

increased LT-tolerance (Vagujfalvi *et al.*, 2000; Francia *et al.*, 2004; Skinner *et al.*, 2005). With the advent of molecular marker technology, it is now possible to delineate the factors contributing to LT-tolerance in wheat and other cereals. The use of molecular markers such as SSRs and AFLPs will help identifying all the regions of the genome that contribute towards LT-tolerance and help to identify markers linked to the LT-tolerance trait. Mapping studies in wheat have shown that the homologous group 5 chromosomes in wheat are not only associated with vernalization, but also contributes substantially towards LT-tolerance. Mapping studies In diploid wheat (*Triticum monococcum*) have revealed two LT-tolerance loci *Fr-A1* and *Fr-A2* located on chromosome 5A (Vagujfalvi *et al.*, 2000; 2003). LT-tolerance loci have also been located on chromosomes 5B and 5D in wheat (Snape *et al.*, 1976; Tóth *et al.*, 2003). Chromosome 5A in wheat encodes transcription factors which are involved in the regulation of *wcs120* and *wcor410* gene families that are mapped to the homologous group 6 chromosomes indicating the chromosome 5A region is also involved in the regulation of genes associated with LT-tolerance. A recent mapping study to identify chromosomal regions implicated in LT-tolerance in a DH population developed from a winter wheat (Norstar) and spring wheat (Manitou) has revealed a major QTL on chromosomes 5A and 1D (Båga *et al.*, 2006a; 2006b). In barley, mapping studies to identify loci associated with LT-tolerance have also indicated that the chromosome 5H in barley which is orthologous to chromosome 5A in wheat has two loci *Fr-H1* and *Fr-H2* that are closely associated with LT-tolerance (Francia *et al.*, 2004; Skinner *et al.*, 2005).

C-repeat binding factors (CBFs) which are transcription factors that regulate the expression of LT-tolerance genes have also been mapped on chromosome 5A in wheat (Miller *et al.*, 2005). Multiple CBF genes have also been reported on barley chromosome 5H (Skinner *et al.*, 2005; 2006).

Due to the large number of genes that are present on the *Vrn-1/Fr-1* chromosome intervals in wheat it is difficult to differentiate the genes involved in LT-tolerance from the vernalization locus. In an attempt to resolve this issue, the main goal of this research project was to differentiate LT-tolerance from vernalization and to identify genomic regions associated with LT-tolerance in winter wheat. A cross between two winter wheat cultivars (Norstar and Cappelle-Desprez) which have the same *vrn-1* region on chromosome 5A (Båga *et al.*, 2006a; 2006b) was utilized in this study. This will help in neutralizing the effect of the *vrn-1* region and may assist in identifying other regions of the genome that are associated with LT-tolerance.

3.2 Materials and Methods

3.2.1 Plant material and growth conditions

Winter wheat *(Triticum aestivum* cv Norstar, cv Capplelle-Deprez) were germinated from seed in plastic pots containing vermiculite in a greenhouse (National Research Council of Canada-Plant Biotechnology Institute, Saskatoon, Saskatchewan) at a temperature of 20/16°C (day/night) with a 16-h photoperiod. Supplemental lighting was provided by fluorescent tubes (Cool White, 160 W,

F72T12/CW/VHO, Sylvania) which was adjusted to a PPFD of 350 µmol m^{-2} s^{-1} at pot height. Water was supplied to all plants as required.

3.2.2 Development and characterization of the DH mapping population

The winter wheat cultivars Norstar and Cappelle-Desprez were used in this study. A previously generated mapping population of 316 DH lines derived from a Norstar X Cappele-Desprez cross was utilized (Båga *et al.*, 2006a; 2006b). The DH lines developed using the Norstar X Cappele-Desprez cross were phenotyped for the LT-tolerance by assessing the plants ability to survive freezing. Freezing tests were conducted by Dr. A. Limin in the laboratory of Dr. D.B. Fowler (Department of Plant Sciences, University of Saskatchewan) according to Limin and Fowler (2002). Norstar has an LT_{50} of -21°C and Cappele-Desprez an LT_{50} of -13°C (Chodaparambil *et al.*, 2004, 2005; Båga *et al.*, 2006a, 2006b).

3.2.3 DNA extraction

Small scale extraction of DNA was performed using a modified method of Doyle and Doyle (1990). Freeze-dried leaves (0.015 g) collected from a 5-10 leaf stage plant were used for DNA extraction. Leaves were ground with ceramic beads along with silica sand in a 2 mL microfuge tube containing 800 µL of extraction buffer (Appendix A). The extraction buffer was heated at 65°C, autoclaved and stored at room temperature. β-mercaptoethanol was added to a final concentration of 20 mM prior to use. The microfuge tubes containing the

ground leaves and the extraction buffer were incubated at 60°C for 10 minutes and 800 µL of chloroform:isoamylalcohol (24:1, v/v) was added followed by centrifugation at 11,000 x g for 3 minutes at room temperature. The clear upper phase was removed to a fresh tube and the chloroform isoamylalcohol extraction step was repeated. The upper phase was transferred to a new microfuge tube, and an equal volume of precipitation buffer (Appendix A) was added. The tubes were gently inverted to allow mixing and incubated at room temperature for 20 minutes. The samples were then centrifuged at 7,000 x g for 10 minutes at room temperature, following which the supernatant was discarded and the pellet obtained suspended in 1 mL of 1 M NaCl. The microfuge tube containing the suspended pellet was heated at 60°C to ensure that the pellet dissolved completely. An equal volume (1 mL) of 100% ethanol was added to the dissolved pellet, mixed well by inverting, followed by incubation at -70°C for 30 minutes and then centrifugation at 11,000 x g for 6 minutes at room temperature. The supernatant was discarded and 1.5 mL of 80% (v/v) ethanol was added to wash the pellet. The pellet was washed and centrifuged for at 11,000 x g for 6 minutes at room temperature. The supernatant was removed with a pipette and the pellet obtained was allowed to air dry. The dried pellet was then dissolved in 150 µL of TE buffer (Appendix A). A total volume of 0.5 µL of 10 mg/mL RNase A was added and the samples were incubated at 37°C for 1 hour to eliminate traces of RNA from the sample. The microfuge tubes were vortexed after adding 150 µL of chloroform:isoamylalcohol (24:1, v/v) and centrifuged at 11,000 x g for 5 minutes at room temperature. The upper phase was transferred to a new

microfuge tube and 1/10th volume of sodium acetate (1.5 M) and 2.5 volumes of ethyl alcohol (100%) were added followed by incubation at -70°C for 15 minutes. The tubes were centrifuged at 11,000 x g for 15 minutes at 4°C to obtain a DNA pellet. The pellet was washed with 80% (v/v) ethanol twice and dissolved in 50 µL TE buffer. DNA was quantified using UV spectrophotometry (Beckman coulter DU800, Fullerton, CA, USA).

3.2.4 Marker analysis

3.2.4.1 Simple Sequence Repeats (SSRs)

SSRs are defined as small segments of DNA, usually 2 to 5 bp in length that repeat themselves a number of times. Some of the major core motifs used in development of SSR markers include ATT, AT, CTT and CT repeats. Useful SSRs usually repeat the core motif 9-30 times. The parent cultivars were tested with SSR primers using a PCR reaction to identify the primers that could reveal polymorphism amongst the parents. The primers which revealed polymorphism in the parents were subsequently used for mapping in the DH lines developed from the Norstar X Cappelle-Desprez cross (Chodaparambil *et al.*, 2004, 2005; Båga *et al.*, 2006a, 2006b). The primer sequences were obtained from publicly available databases (http://wheat.pw.usda.gov; http://www.scabusa.org) and included 239 gwm markers, 168 wmc markers, 161 barc markers, 72 cfd markers, 20 cfa markers, 10 gdm markers and 8 gpw markers. Only markers determined to be polymorphic in the parent cultivars were used for mapping in the DH lines (Chodaparambil *et al.*, 2004, 2005; Båga *et al.*, 2006a, 2006b).

3.2.4.2 PCR amplification using SSR primers

A standard PCR reaction consisted of DNA (25 ng), dNTPs (200 µM of dATP, dCTP, dGTP, dTTP each), forward and reverse primers (10 pmol each), and 1 U of *Taq* DNA polymerase (Cat. No- D4309; Sigma, St, Louis, MO, USA) in a PCR reaction buffer (Appendix A) and sterile H_2O to a total volume of 25 µL. A MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) was used for amplification of the template DNA. The PCR conditions were as follows: a 4 minute hold at 94°C, followed by 40 cycles of denaturing at 94°C for 30 seconds, annealing (temperature varied from 55°°C to 65°C, depending on the primers used) for 45 seconds, and extending at 72° for 2 minutes. A final extension at 72°C for 10 minutes was done for each reaction.

The amplified products were analyzed by agarose gel electrophoresis. Sigma REDTaq DNA polymerase used for amplification of genomic DNA was directly loaded on the agarose gel after the completion of PCR without the addition of a loading buffer or tracking dye. The red tracking dye present in the REDTaq DNA polymerase migrates with the same rate as a 125 bp fragment. Separation occurred in a 2.0% (w/v) gel electrophoresed at 106 V/cm at constant voltage in a horizontal gel apparatus (Gibco BRL). Twenty µg of ethidium bromide was added directly to the gel for subsequent visualization. TAE (Appendix A) was used as a running buffer. The amplified fragments were visualized under UV light using a gel documentation apparatus (Chemi-Doc XRS,

Bio-Rad). Quantity One software (Bio-Rad) was used to determine the sizes of the amplification products.

The amplified products that could not be resolved on an agarose gel were analysed by polyacralamide gel electrophoresis (PAGE). Amplified DNA products were separated on a 6% (w/v) denaturing polyacrylamide gel. The glass plates used for PAGE were washed with distilled-water and wiped with 70% (v/v) ethanol. One glass plate was treated with a binding solution (Appendix A) which helps to chemically cross-link the gel to the glass plate. The other glass plate was treated with 3 mL of Sigmacote (Sigma) solution which assists separating the glass plates. The treated plates used for electrophoresis were separated with 0.4 mm spacers and clamped tightly. The gel was prepared using 6% (w/v) acrylamide, 0.25% (w/v) methylene bisacrylamide, 0.5X TBE buffer (Appendix A) and 6 M urea (pH 8.0). A 3 µL aliquot of the PCR product was mixed with 2x loading buffer (Appendix A) and denatured at 95°C for 2 minutes before loading in the gel. The gel was run at constant wattage (80 W) for 1 hour on a Gibco-BRL S2 sequencing apparatus using 0.5X TBE as a running buffer. After electrophoresis the glass plates were separated carefully. The gel (still attached to one plate) was placed in a tray with fix/stop solution (Appendix A) on an orbital shaker set at 30 rpm for 20 minutes. The gel was rinsed 3 times (2 minutes each) in distilled water and then silver stained using a staining solution (Appendix A) for 30 minutes on an orbital shaker set at 30 rpm. The gel was rinsed with distilled water for 10 seconds and transferred to a tray containing developer solution (Appendix A) until the appearance of bands. Once the bands appeared on the

gel a stop solution (Appendix A) was added to stop the reaction. The gel was washed in distilled water and allowed to dry overnight. Sizes of the PCR products were determined using a gel documentation system (Chemi Doc XRS, Bio-Rad) and software provided by the manufacturer (Quantity One, Bio-Rad).

3.2.5 PCR amplification using AFLP primers

3.2.5.1 Restriction digestion of genomic DNA and ligation of adapters

The AFLP procedure was performed as described by Vos *et al.* (1995). In this procedure, the genomic DNA is digested with a 6 bp restriction enzyme (*Eco*RI, a 'rare cutter') and a 4 bp restriction enzyme (*Mse*I, a 'frequent cutter'). Adaptors of known sequences are ligated to the ends of the fragments generated by the two restriction enzymes. A "pre-selective amplification" of the template DNA is performed by using the adaptor sequences for hybridization of the primers complementary to the *Eco*RI and *Mse*I ends. A "selective amplification" is then performed using the products obtained after pre-selective amplification as a template and by the use of primers containing one or more additional bases at the 3'-end in a PCR reaction to obtain the final products that will be loaded on the gel. The bases at the 3'-end of the primer have a marked effect on the final AFLP fingerprint as a single 3'-extension reduces the number of fragments generated by a factor of 16, a two-base extension by a factor of 256 and a three-base extension reduces the number by 4,096 Vos *et al.* (1995).

Restriction digestion of genomic DNA (500 ng) and ligation of adapters was carried out in a single reaction tube. The adapters and primers used in this

study are listed in Table 3.1. The *Eco*RI and *Mse*I adaptor pairs were heated to 95°C for 5 minutes, separately, and allowed to cool slowly to room temperature before being added to the digested DNA. The restriction digestion and ligation reaction were incubated overnight at room temperature. The digested-ligated DNA was diluted 1:18 in TE buffer.

Table 3.1 AFLP primers and adapter sequences used for AFLP analysis in the Norstar X Cappelle-Desprez cross

Primers/adapters	*Sequences*
*Mse*I adapter	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
M00 (universal primer)	GATGAGTCCTGAGTAA
*Mse*I + 1	M00 + C
*Mse*I + 3-primers	
M47	M00 + CAA
M48	M00 + CAC
M49	M00 + CAG
M50	M00 + CAT
M59	M00 + CTA
M60	M00 + CTC
M61	M00 + CTG
M62	M00 + CTT
*Eco*RI adapter	5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
E00 (universal primer)	GACTGCGTACCAATTC
*Eco*RI + 1	E00 + A
*Eco*RI + 3-primers	
E32	E00 + AAC
E33	E00 + AAG
E36	E00 + ACC
E37	E00 + ACG
E40	E00 + AGC
E41	E00 + AGG

3.2.5.2 Pre-selective amplification of template DNA

Pre-selective amplification occurred in a reaction containing 4 µL of the digested/ligated DNA, 2 µL of PCR reaction buffer, dNTPs (200 µM of dATP, dCTP, dGTP, dTTP each), 1 U *Taq* DNA polymerase, 0.043 µL of *Eco*RI + 1 (300 ng/µL), 0.044 µL of *Mse*I + 1 (300 ng/µL) pre-selective primers and sterile H_2O to a volume of 20 µL. The PCR pre-amplification conditions were as follows: 72°C for 3 minutes, followed by 20 cycles of denaturing at 94°C for 20 seconds, annealing at 56°C for 30 seconds, and extending at 72°C for 2 minutes, which was followed by a final hold at 60°C for 30 minutes. All samples were amplified using a MyCycler thermal cycler (Bio-Rad). The pre-selective amplification product was diluted 20-fold using TE buffer.

3.2.5.3 Selective amplification

Selective amplification occurred in a reaction containing 3 µL of diluted pre-amplified template DNA, 2 µL of PCR reaction buffer, dNTPs (200 µM of dATP, dCTP, dGTP, dTTP each), 1 U *Taq* DNA polymerase, *Eco*RI primer (*Eco*RI+3-primer) 0.2 µL (30 ng/µL), *Mse*I primer (*Mse*I+3-primer) 1.0 µL (30 ng/µL) with two additional nucleotides and sterile H_2O to a volume of 20 µL. All of the *Eco*RI and *Mse*I primer combinations were tested for amplifcation and only those demonstrating sucessfull amplification profiles were used (Table 3.2). All samples were amplified using a MyCycler thermal cycler (Bio-Rad). The selective amplification conditions were as follows: 94°C for 2 minutes, followed by 1 cycles of denaturing at 94°C for 20 seconds, annealing at 66°C for 30 seconds, and extending at 72°C for 2 minutes, followed by 10 subsequent cycles. In each cycle the temperature was reduced by 1°C down to 56°C. This was

followed by 25 cycles of denaturing at 94°C for 20 seconds, annealing at 56°C for 30 seconds and extending at 72°C for 2 minutes, which was followed by a final hold at 60°C for 30 minutes.

Table 3.2 *EcoR*I and *Mse*I primer combinations used for selective amplification.

Primer combination	*Primer combination*	*Primer combination*
E32 + M47	E36 + M60	E40 + M59
E32 + M48	E36 + M61	E40 + M60
E32 + M49	E36 + M62	E40 + M61
E32 + M50	E37 + M47	E40 + M62
E32 + M59	E37 + M48	E41 + M48
E32 + M62	E37 + M49	E41 + M49
E33 + M47	E37 + M50	E41 + M49
E36 + M47	E37 + M59	E41 + M50
E36 + M49	E37 + M60	E41 + M59
E36 + M50	E37 + M61	E41 + M62
E36 + M59	E37 + M62	

3.2.5.4 Detection of AFLP markers

The PCR products were separated, visualized and their sizes determined as described in section 3.2.4.2.

3.2.6 Linkage and QTL analysis

The segregation of the polymorphic band(s) (marker data) in the DH population was entered as raw data in the JoinMap 3.0 software (Van Ooijen and Voorrips, 2001). The linkage map was constructed using JoinMap 3.0 software for the calculation of genetic linkage maps (Van Ooijen and Voorrips, 2001). To identify the linkage groups the Logarithmic of Odds (LOD) score was set from 2.0 to 10. Initial linkage groupings of the makers were identified from the highest LOD score values starting from LOD 10. Markers that did not belong to the initial

linkage group at LOD ≤ 3.0 were not included in the analysis. Linked SSR and AFLP markers were grouped together based on the linkage results obtained using the JoinMap software. The marker order and the chromosomes to which the SSR markers mapped in the Norstar X Cappelle-desprez cross was compared to other published maps. New markers identified in this mapping population were considered unique if the fragment size obtained by the markers was not published in other maps and also if different fragment sizes obtained using SSR or AFLP markers mapped to a different position or chromosome not reported in other published maps. Recombination frequencies calculated between the markers by the JoinMap software are a measure of genetic linkage and is the fundamental criteria used in the assembly of a linkage or genetic map. Recombination frequencies were converted into centiMorgan (cM) map distances using the Kosambi mapping function (Kosambi, 1944).

The JoinMap MapQTL program was used to detect QTLs (Van Ooijen, 2004). Putative QTLs were identified using the Kruskal-Wallis rank sum test. Interval mapping identified the major QTLs in the Norstar X Cappelle-Desprez mapping population using forty DH lines ranging from low to high LT_{50} values. The critical LOD threshold value in declaring the presence of a QTL was determined by the 1,000 permutation test using the 40 DH lines and was set at $P = 0.05$ (Churchill and Doerge, 1994).

3.3 Results

3.3.1 LT-tolerance of the DH lines selected for mapping

A DH population of 316 lines was developed from the Norstar X Cappelle-Desprez cross (Båga *et al.*, 2006a, 2006b) and utilized in this study. The DH lines had LT_{50} values in a continuous distribution ranging from -21°C to -13°C indicating the LT-tolerance trait is polygenic and quantitative (Båga *et al.*, 2006a) (Fig. 3.1A). None of the DH lines in the population developed from the Norstar X Cappelle-Desprez cross significantly exceeded Norstar's LT-tolerance.

For constructing a linkage map of Norstar X Cappelle-Desprez, 40 DH lines were used. Twenty-one lines were termed as hardy lines ($LT_{50} \geq$ -16° C) and nineteen lines were termed as less-hardy ($LT_{50} \leq$ -16° C) based on the LT_{50} values (Chodaparambil *et al.*, 2004, 2005; Båga *et al.*, 2006a, 2006b) (Fig. 3.1B).

3.3.2 Norstar X Cappelle-Desprez linkage map assembly

3.3.2.1 SSR and AFLP marker analysis

To assemble a linkage map of Norstar X Cappelle-Desprez a total of 625 SSR and 64 AFLP markers were used to test for polymorphism among the parent lines (Norstar and Cappelle-Desprez). Representative gels are shown in Figs. 3.2 and 3.3. Three hundred and thirty-two markers were polymorphic using SSRs while 28 primer combinations revealed polymorphisms using the AFLP marker system (Table 3.3). A total of 443 loci were amplified using SSRs while the AFLP marker system amplified 197 loci (Table 3.3). Using the SSR marker system a total of 147 markers were mapped to the A genome, 149 markers were mapped to the B genome and 135 markers were mapped to the D genome (Table 3.3). The AFLP primer combinations mapped 70 markers to the A

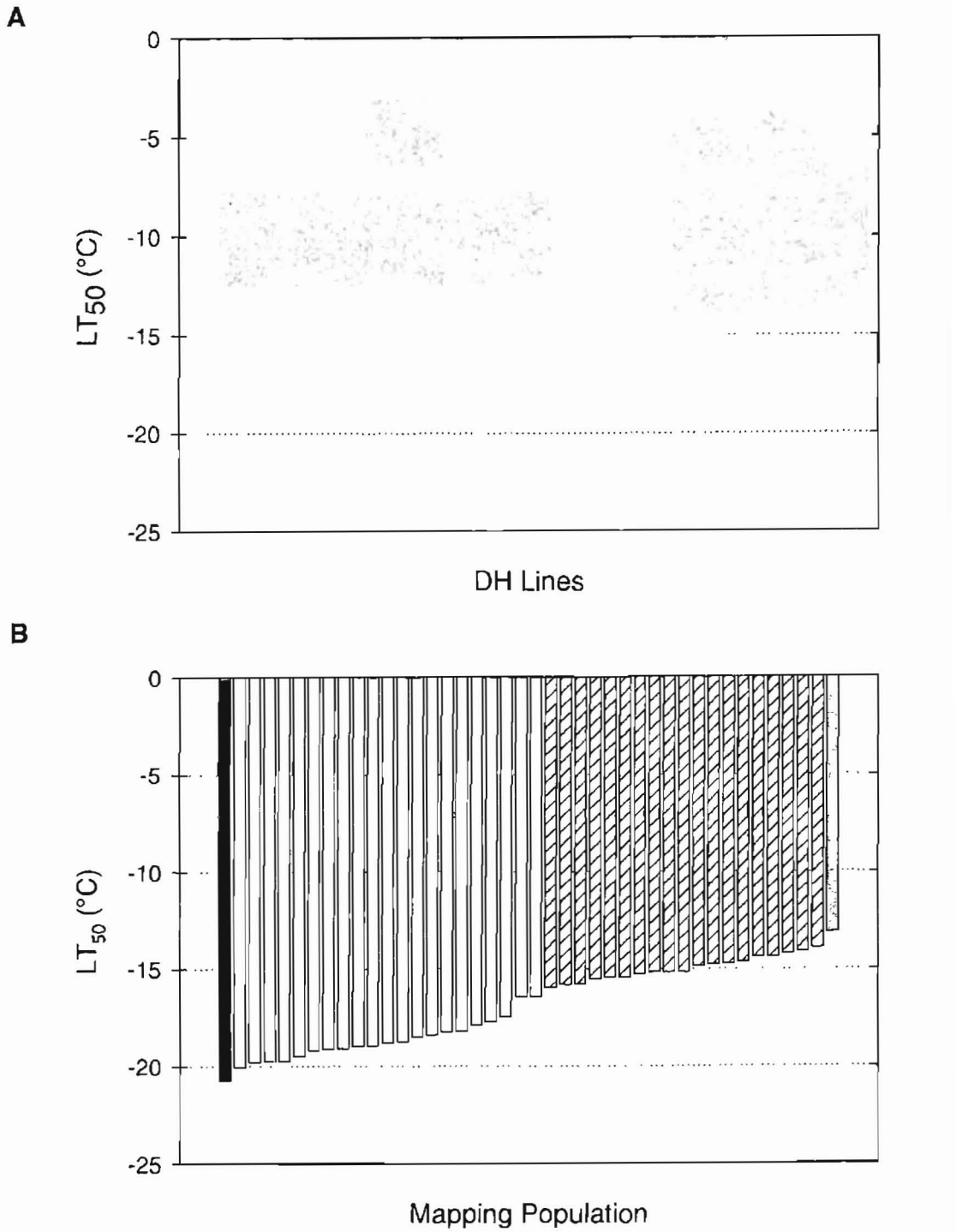


Figure 3.1 LT_{50} values of DH lines. (**A**) LT_{50} values for all of the DH lines obtained from the Norstar X Cappelle- Desprez cross (Båga *et al.*, 2006a). (**B**) LT_{50} values of the hardy and less-hardy lines used for constructing the linkage map of Norstar X Cappelle-Desprez presented in Figure 3.4 (Chodaparambil *et al.*, 2004, 2005; Båga *et al.*, 2006a). Norstar, (■); hardy lines, (□); less-hardy lines, (▨); Capelle-Desprez, (□). Figure modified from Chodaparambil *et al.* (2005) and Båga *et al.* (2006a)

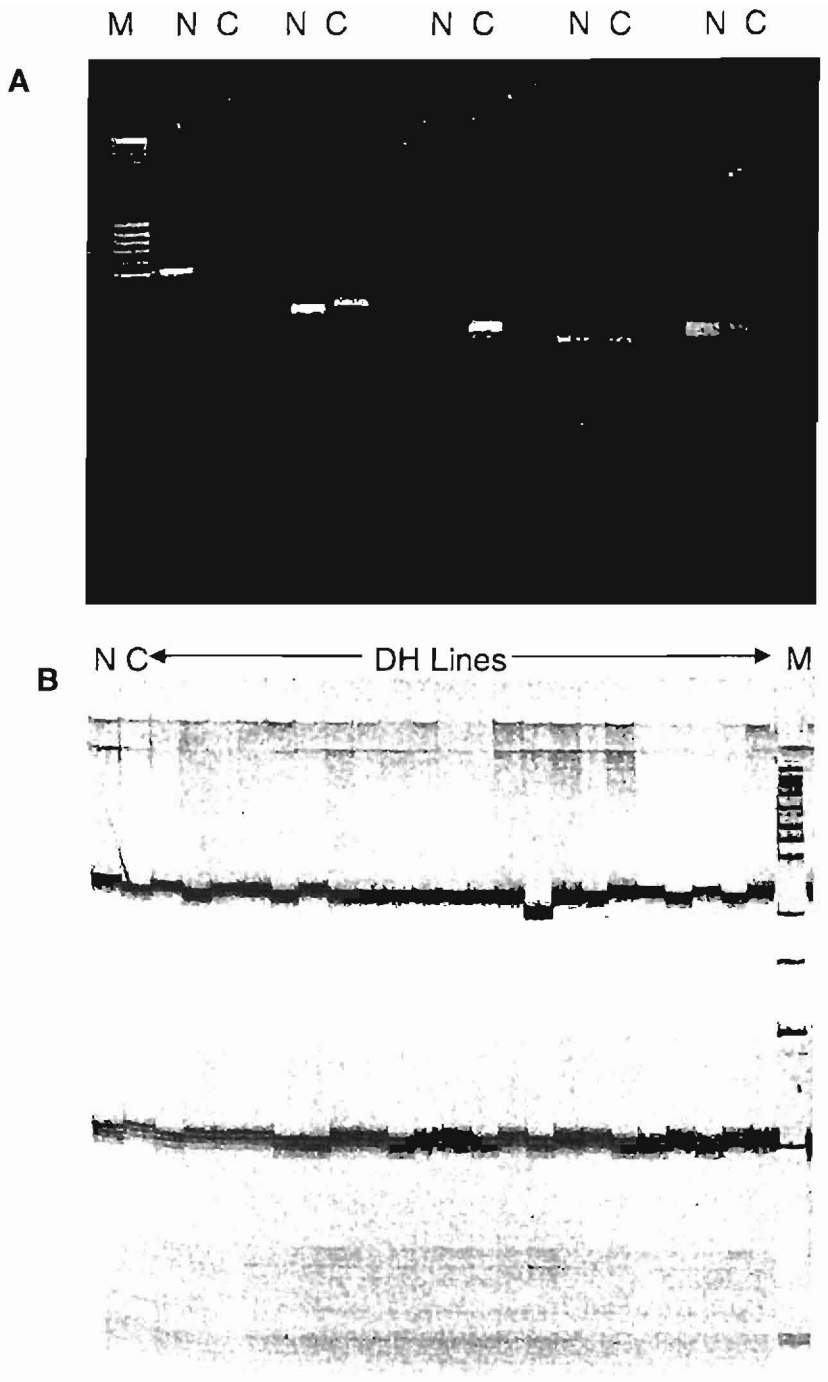


Figure 3.2 SSR marker analysis. (**A**) Agarose gel electrophoresis of N, Norstar and C, Cappelle-Desprez DNA analyzed using five SSR primer pair in a PCR reaction; M, marker (**B**). Polyacrylamide gel electrophoresis of amplified DNA products obtained after performing a PCR reaction using a SSR primer pair with DNA from N, Norstar; C, Cappelle-Desprez; DH lines, double haploid lines obtained using the Norstar X Cappelle-Desprez cross. A representative photograph is shown.

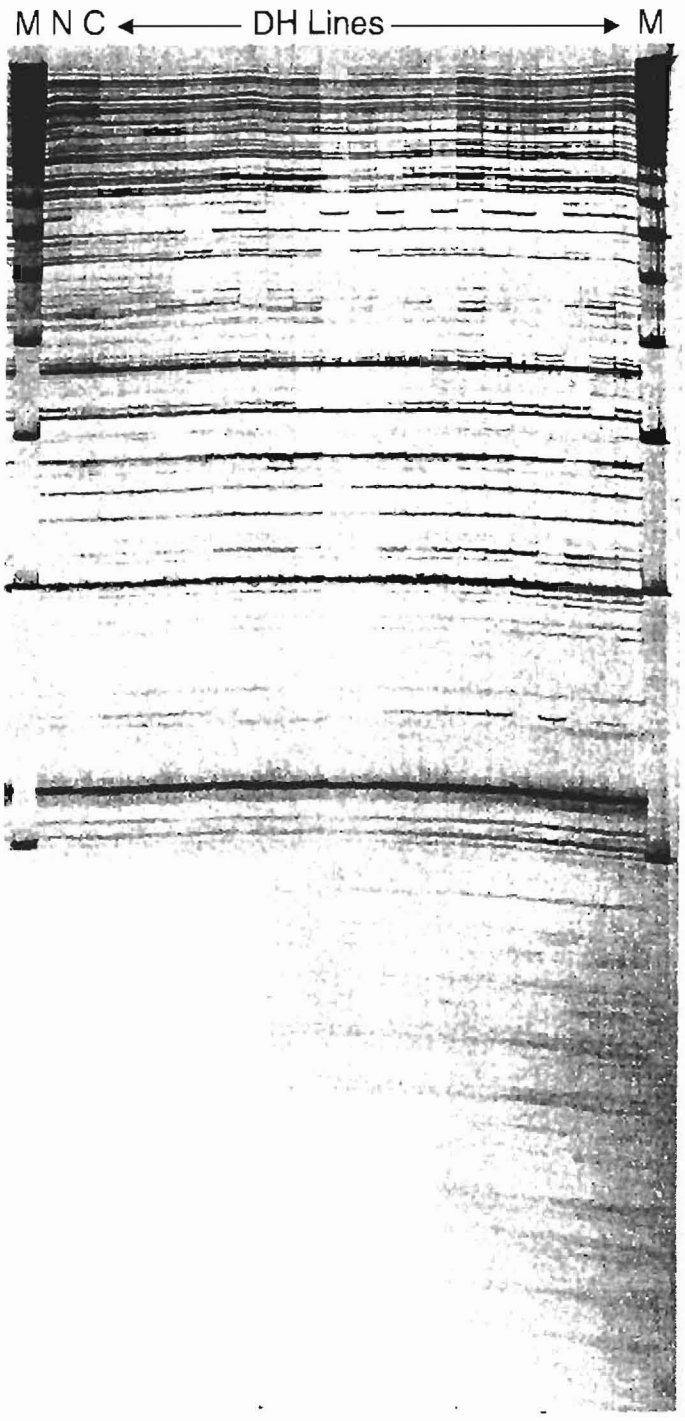


Figure 3.3 PAGE analyses. Amplified DNA products after performing PCR with DNA obtained from N, Norstar; C, Cappelle-Desprez; DH lines, double haploid lines obtained using the Norstar X Cappelle-Desprez cross using the AFLP primer combination E40+M60. M, marker; A representative photograph is shown.

genome, 88 markers to the B genome and 30 markers to the D genome (Table 3.3). Therefore, using both the SSR and AFLP marker systems a total of 360 primer combinations amplified 640 loci of which 217 markers mapped to the A genome, 237 markers mapped to the B genome and 165 markers mapped to the D genome as indicated in Table 3.3.

Table 3.3. SSR and AFLP markers mapped. The markers mapped to different genomes (A, B and D) are also shown.

Primer	*Primers screened*	*Polymorphic*	*Loci amplified*	*Genome (markers mapped)*			*Unlinked*
				A	*B*	*D*	
Gwm	248	119	153	50	57	44	2
Wmc	145	89	127	45	47	32	3
Barc	159	76	100	34	35	31	0
Cfd	40	29	38	6	7	21	4
Cfa	17	12	13	9	1	0	3
Gdm	8	5	10	3	0	7	0
Gpw	8	2	2	0	2	0	0
AFLP	64	28	197	70	88	30	9
Total	689	360	640	217	237	165	21

The AFLP and microsatellite marker data was analyzed using Joinmap 3.0 software. In the Norstar X Cappelle-Desprez DH population, 40 DH lines were used in the analysis to assign 640 loci to 44 linkage groups while 21 loci could not be assigned to any linkage group and were considered as unlinked (Table 3.3). Markers not determined in the present study were removed after the initial linkage analyses. To group the markers, a LOD score of ≥ 3.0 was used and the linkage group contained at least three markers. Highly distorted loci ($P < 0.005$) were eliminated from the Norstar X Cappelle-Desprez population because the segregation distortion will affect the recombination ratios. Analysis of the

segregation ratio for each marker locus from expected values by a chi-square goodness-of-fit test indicated a segregation distortion of 1.8% in the Norstar X Cappelle-Desprez cross. The marker density on the chromosomes varied from 2.2 cM/marker on chromosome 5B to 8.2 cM/marker on 5D (Table 3.4). Linkage groups obtained in the Norstar X Cappelle-Desprez cross ranged from 1 linkage group in 1B, 2A, 2D, 4B and 5D chromosomes to a maximum of 4 linkage groups present in chromosome 7A (Table 3.4). The presence of more than 1 linkage group in a chromosome is an indication of a lack of polymorphism between the parents for the markers tested or due to the absence of polymorphism in those genomic regions.

The linkage map assembled using a DH population derived from Norstar X Cappelle-Desprez has a total genetic length of 2292 cM and comprises 443 SSR markers and 197 AFLP markers (Fig 3.4). Chromosome 7A had the maximum number of linkage groups (four), while chromosomes 1B, 2A, 2D, 4B and 5D have one linkage group each. The markers identified to be associated with LT-tolerance trait have been indicated in the linkage map of Norstar X Cappelle-Desprez (Fig 3.4).

3.3.2.2 Comparison of SSR markers with other maps.

Comparison of the 443 loci mapped using SSR markers in the Norstar X Cappelle-Desprez cross with other published maps such as Synthetic X Opata (Roder *et al.*, 1998), Consensus map (Somers *et al.*, 2004), and Composite map (http://wheat.pw.usda.gov), has shown that the markers mapped to various

Table 3.4. Linkage groups and marker density. Number of markers present on each chromosome and genetic length in cM are also indicated.

Chromosome	*No.of markers*	*Genetic length (cM)*	*Linkage groups*	*Marker density (cM/marker)*
1A	24	96 (76+20)	2	4.0
1B	32	118	1	3.6
1D	17	92 (26+66)	2	5.4
2A	25	109	1	4.3
2B	32	132 (71+56+5)	3	4.1
2D	35	130	1	3.7
3A	19	128 (80+48)	2	6.7
3B	43	141 (101+40)	2	3.2
3D	26	95 (20+15+60)	3	3.6
4A	21	99 (57+42)	2	4.7
4B	23	61	1	2.6
4D	18	50 (22+28)	2	2.7
5A	45	131 (98+15+18)	3	2.9
5B	44	97 (27+19+51)	3	2.2
5D	24	198	1	8.2
6A	25	125 (107+17)	2	5.0
6B	30	92 (85+7)	2	3.0
6D	16	95 (64+31)	2	5.9
7A	41	104 (8+11+39+46)	4	2.5
7B	25	77 (19+58)	2	3.0
7D	22	122 (77+38+7)	3	5

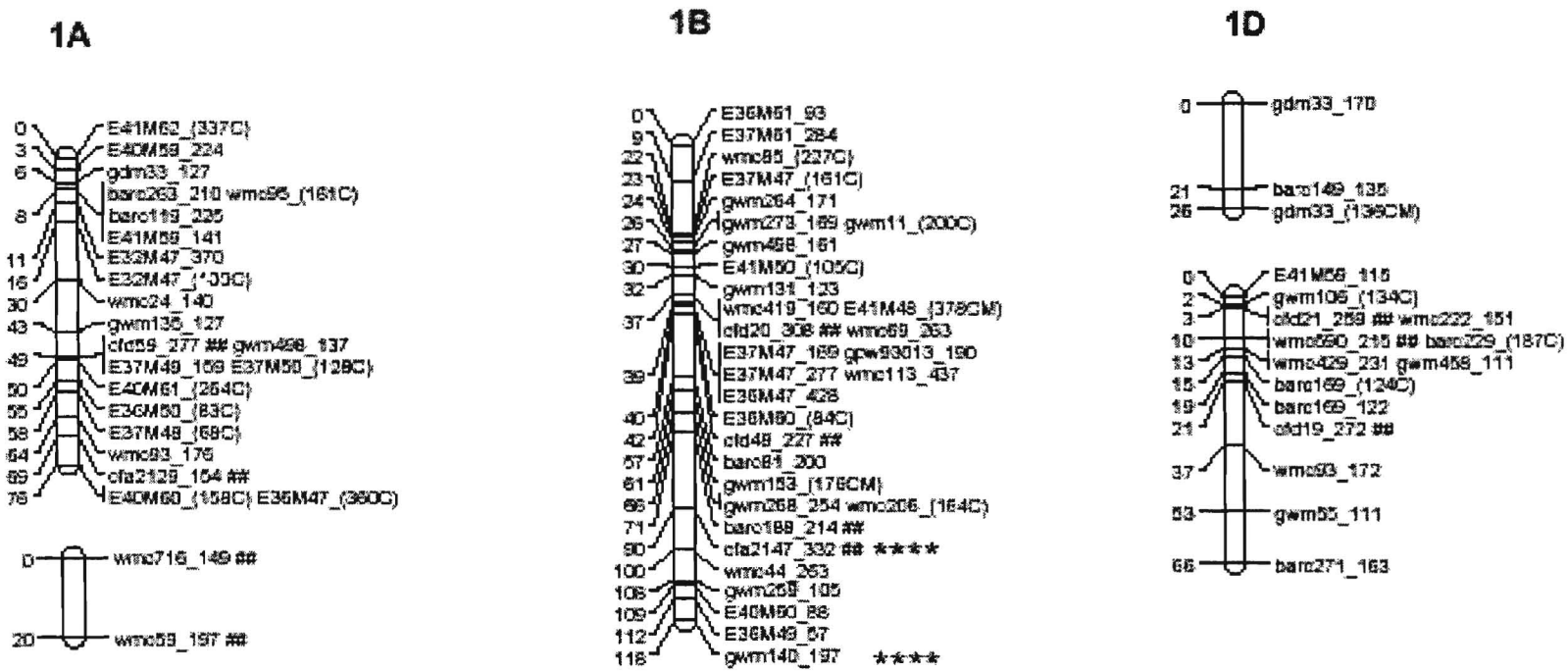


Figure 3.4 Genetic linkage map of the Norstar X Capplle-Desprez cross. Markers are shown on group 1, group 2, group 3, group 4, group 5, group 6 and group 7 chromosomes as indicated. Marker position is indicated in Kosambi cM and the allele sizes are indicated beside the marker name (marker name_ marker size) in bp. Kruskal-Wallis test was used to identify markers associated with LT-tolerance and have been indicated by symbols [**** (P<0.005), ***** (P<0.001), ****** (P<0.0005), and ******* (P<0.0001)] based on their significance. All markers presented in this Figure, with the exception of those indicated by ##, were determined in this study. ## Markers not determined in this study have been presented previously (Båga et al., 2006b).

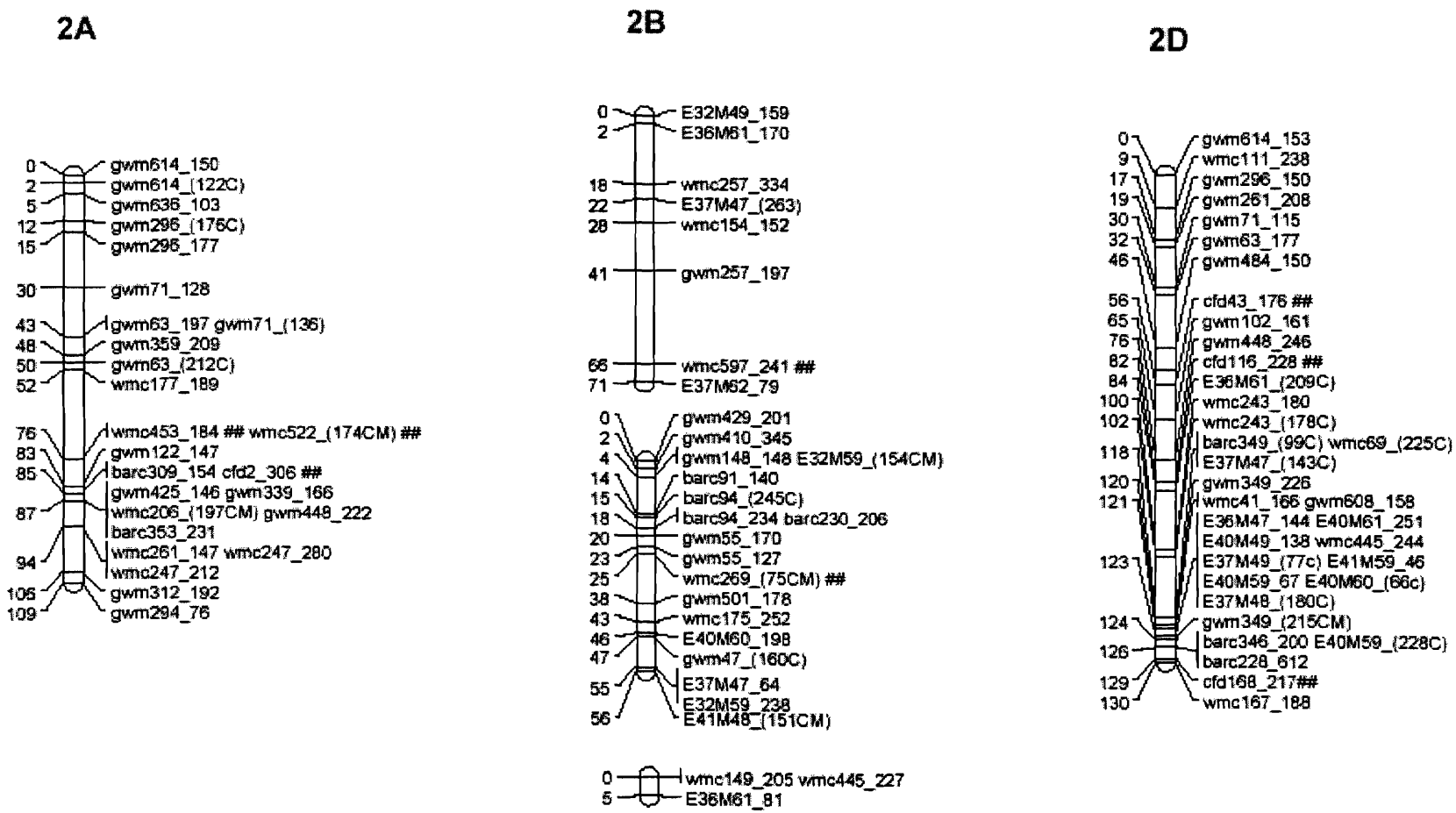


Figure 3.4 Genetic linkage map of the Norstar X Cappile-Desprez cross (continued).

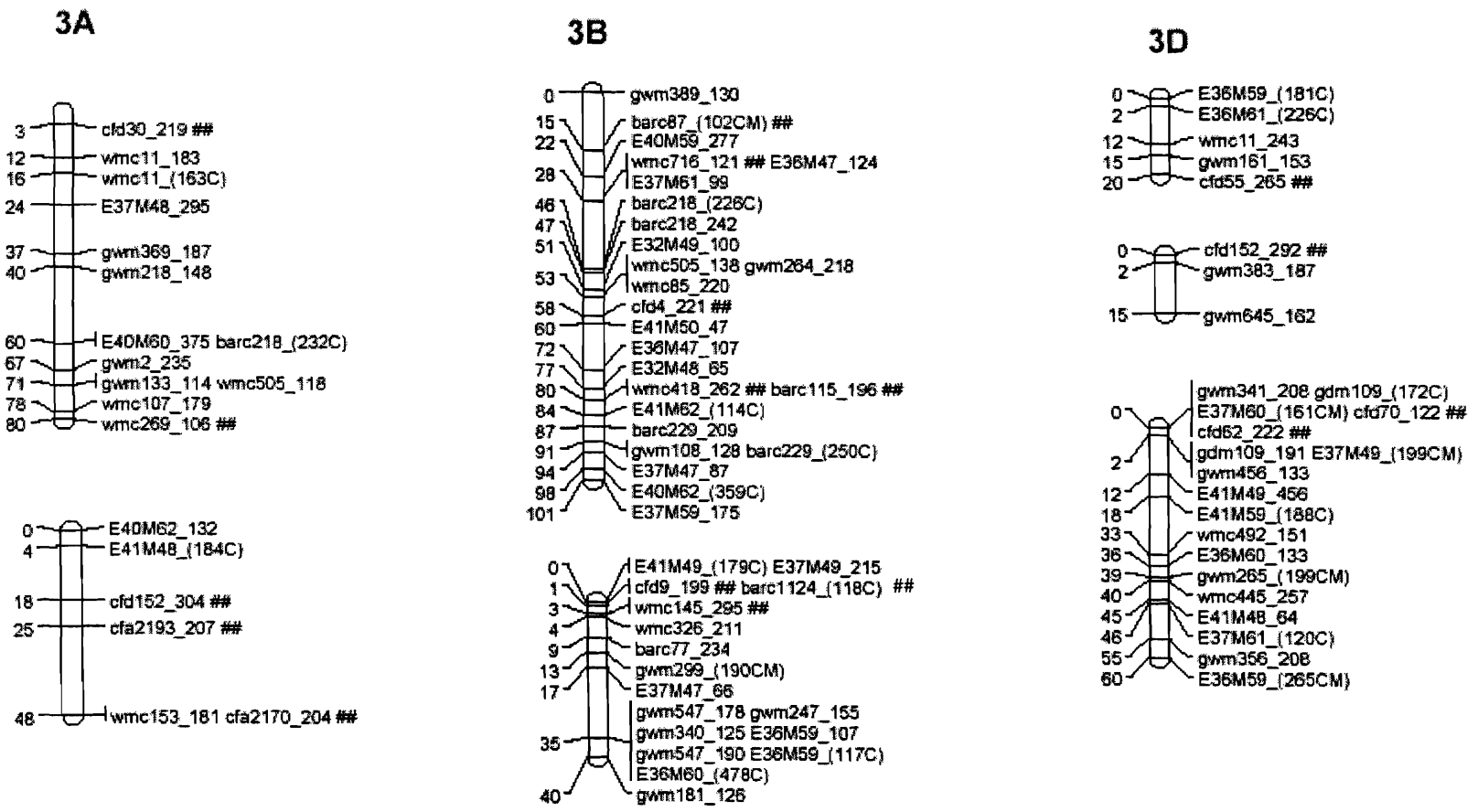


Figure 3.4 Genetic linkage map of the Norstar X Capplle-Desprez cross (continued).

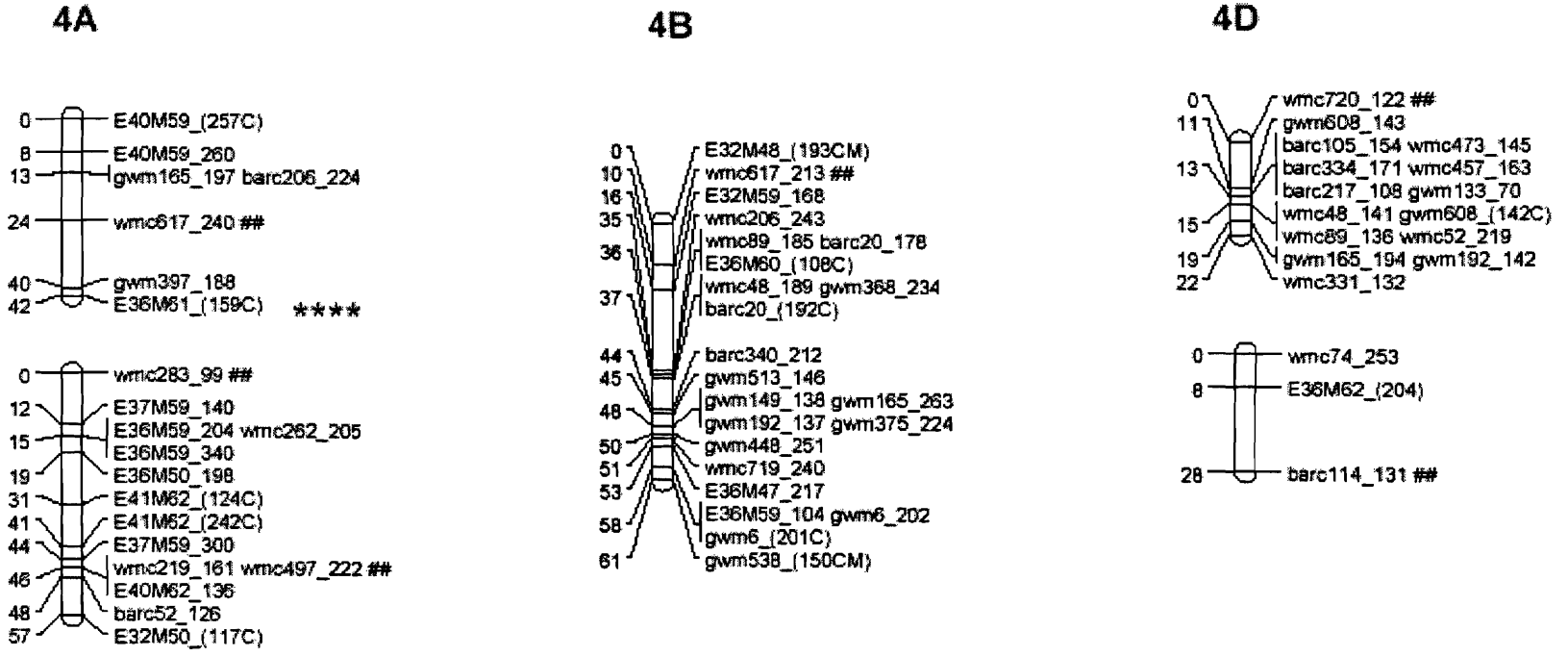


Figure 3.4 Genetic linkage map of the Norstar X Capplle-Desprez cross (continued).

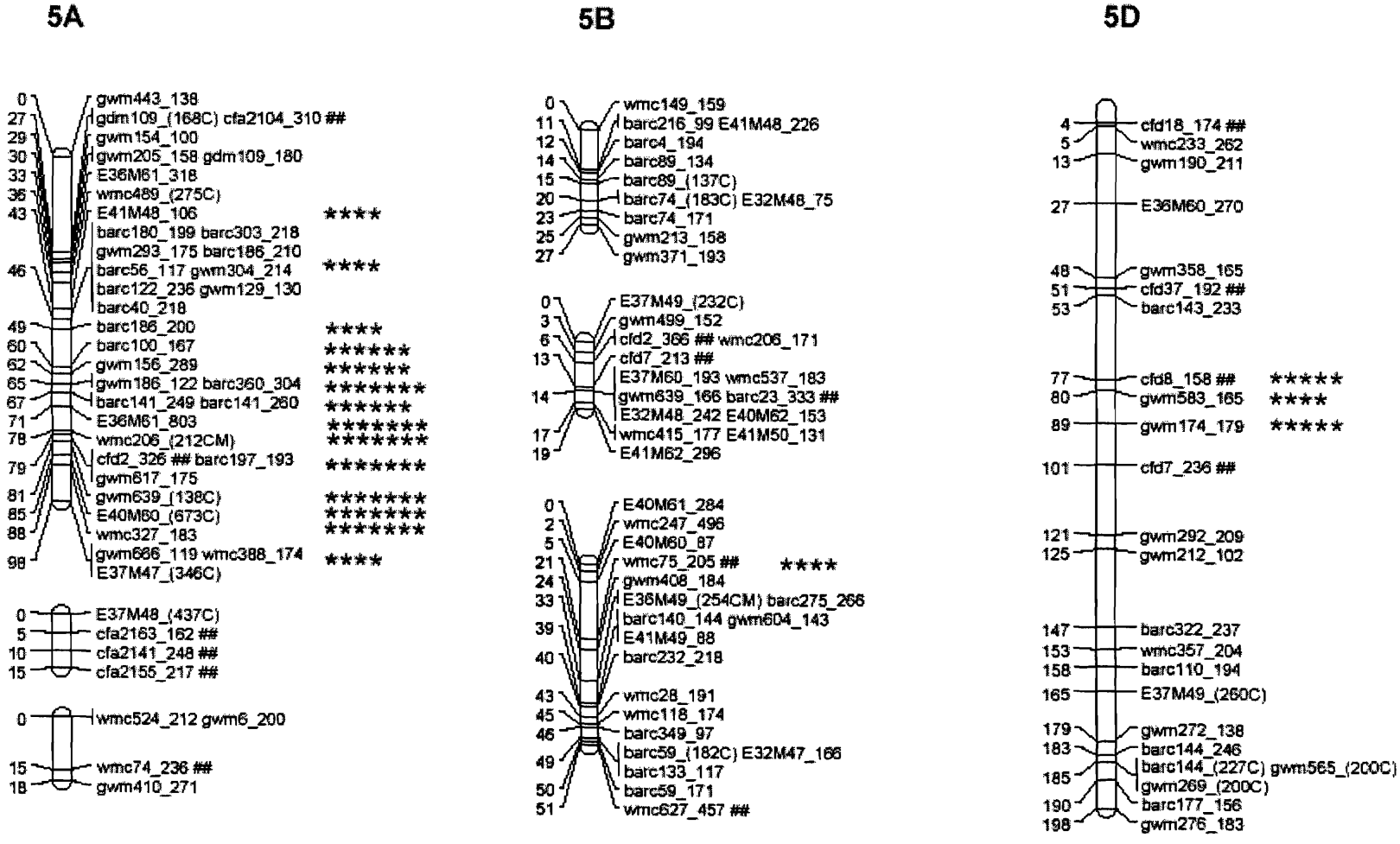


Figure 3.4 Genetic linkage map of the Norstar X Capplle-Desprez cross (continued).

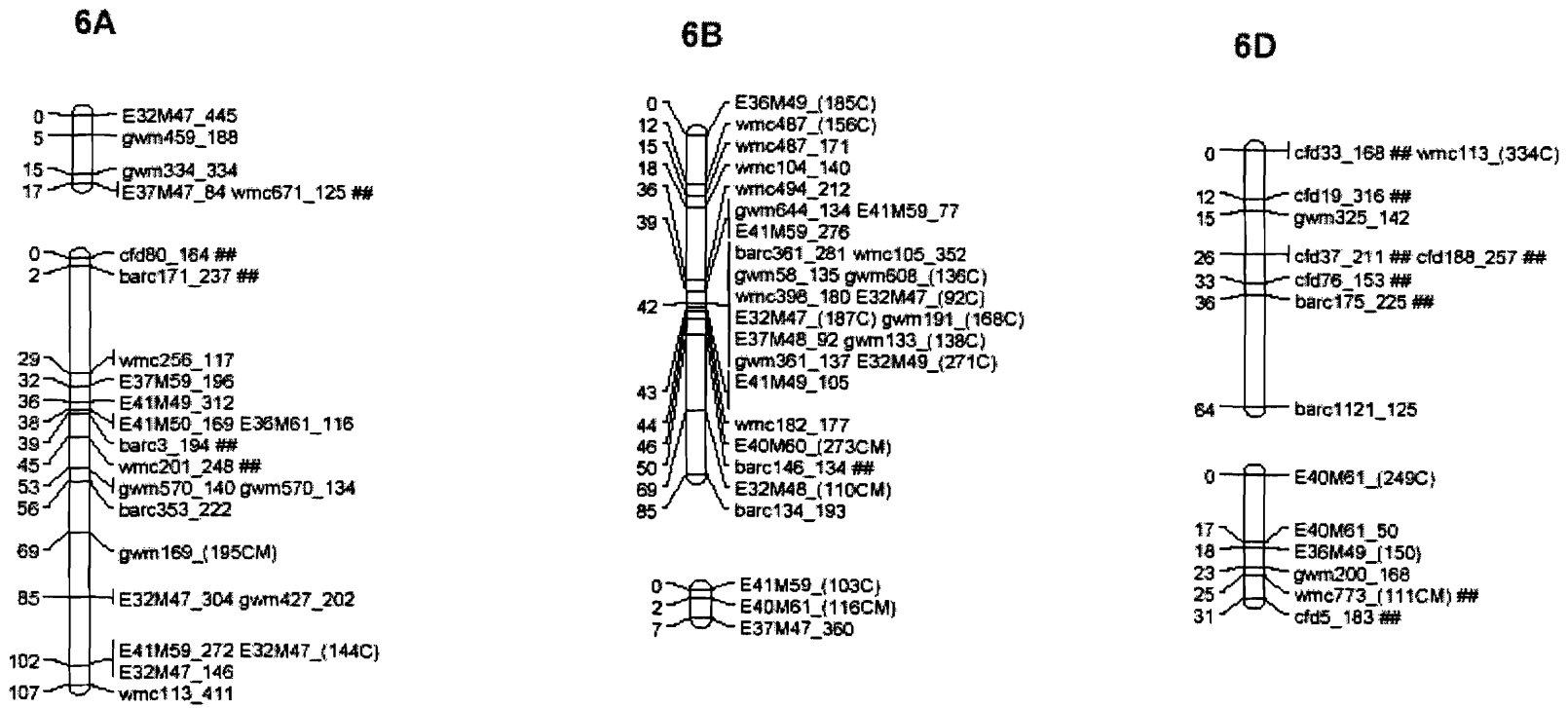


Figure 3.4 Genetic linkage map of the Norstar X Capplle-Desprez cross (continued).

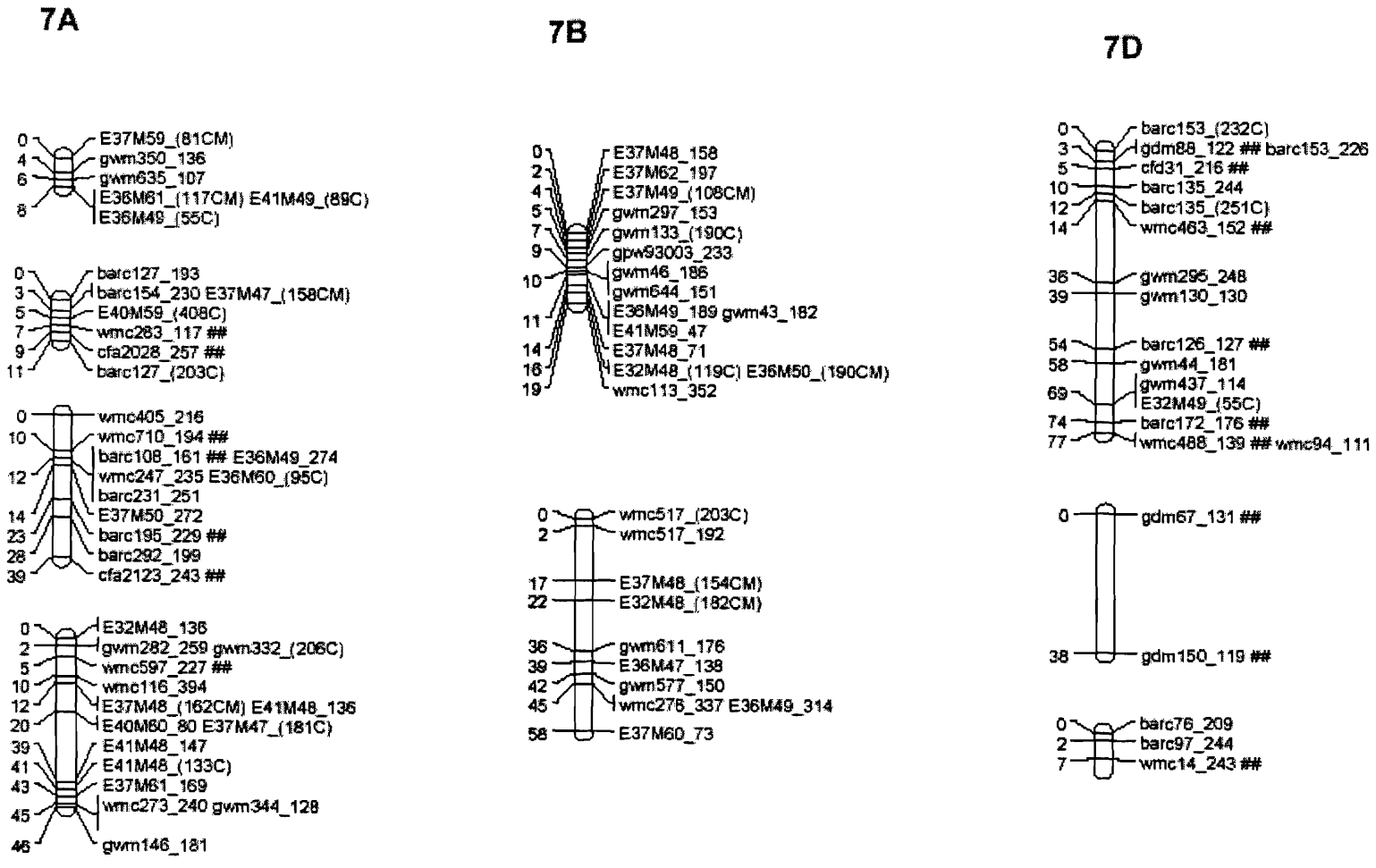


Figure 3.4 Genetic linkage map of the Norstar X Capplle-Desprez cross (continued).

chromosomes in the Norstar X Cappelle-Desprez cross are in agreement with other published maps (Appendix B).

3.3.2.3 Unique SSR and AFLP markers

Sixty-nine unique SSR markers were mapped on A genome (29 markers), B genome (18 markers) and D genome (14 markers) respectively. The unique markers mapped in the Norstar X Cappelle-Desprez cross have not been previously mapped in any mapping population. The locations of the unique SSR markers mapped in the Norstar X Cappelle-Desprez cross are shown in (Appendix C).

A total of 197 loci were amplified using 28 AFLP primer combinations. All the AFLP markers reported in the Norstar X Cappelle-Desprez crosss are unique and have not been reported in any published maps. (Appendix D) indicates the chromosome locations of the mapped AFLP markers. The markers which were not mapped and did not belong to any linkage groups have been indicated as unlinked. Using the 28 AFLP primer combination resulted in mapping of 70 AFLP markers to the A genome, 88 markers to the B genome and 30 markers to the D genome while nine markers remained unlinked (Table 3.5).

Therefore, a total of 249 unique SSR and AFLP markers were mapped to the A genome (99 markers), B genome (106 markers) and D genome (44 markers) as shown in the Table 3.5.

Table 3.5 Distribution of unique SSR and AFLP markers

Primer	*Loci amplified*	*Genome (markers mapped)*			*Unlinked*
		A	*B*	*D*	
Gwm	20	8	3	8	1
Wmc	25	14	9	1	1
Barc	11	3	5	3	0
Cfd	4	3	1	0	0
Cfa	2	1	0	0	1
Gdm	2	0	0	2	0
Gpw	0	0	0	0	0
AFLP	197	70	88	30	9
	Total	99	106	44	12

3.3.2.4 Identification of markers linked with LT-tolerance

MapQTL5 software (Van Ooijen, 2004) was used to determine markers associated with putative LT-tolerance QTLs in the Norstar X Cappelle-Desprez cross. Kruskal-Wallis rank sum test (K) was performed based on the phenotypic and genotypic data from 40 DH lines used as the mapping population to identify markers associated with the trait (LT_{50}) data (Chodaparambil *et al.*, 2005; Båga *et al.*, 2006a). A LOD score value of 3.8 was determined as a significant threshold value for identification of putative QTLs associated with LT-tolerance using simulation tests in the MapQTL5 software. A P value ≤ 0.01 was considered significant for identification of a putative QTL. Chromosomes 1B, 1D, 4A, 5A, 5B and 5D comprised regions associated with LT-tolerance as shown in the Table 3.6. The markers identified to be associated with LT-tolerance trait have been indicated in the linkage map of Norstar X Cappelle-Desprez (Fig 3.4).

All the putative QTLs identified using the Kruskal-wallis test (K) were based on multiple markers except on chromosome 4A, which had only 2

markers. The Kruskal-wallis test is a non-parametric ANOVA used to estimate the significance of differential expression between two or more samples or groups. The putative QTL on chromosome 1B showed a region from 90 to 112 cM associated with the LT-tolerance trait with the marker cfa2147_332 showing the highest K value (10.2). Chromosome 1D had 3 markers in a region covering 9 cM with K values of 7.2 each. On chromosome 4A two markers were identified with K values of 6.9 (gwm397_188) and 9 (E36M61_159C) respectively. Chromosome 5A indicated highest K-values for a putative QTL spanning a region from 43 to 98 cM. The markers with the highest K-values on the 5A chromosome are cfd2_326, barc197_193, gwm617_175 all with a K-value of 26.8 and gwm639_138C with a K-value of 26.9. All of the above markers have a significant *P* value of 0.0001. A putative QTL with a *P* value of 0.01 was also located on chromosome 5B (21 to 40 cM region) with the peak marker wmc75_205 having a K-value of 8.4. On chromosome 5D a region from 77 to 89 cM indicated a putative QTL with markers having K-values of 11.6 (cfd8_158), 9.9 (gwm583_165), 11.9 (gwm174_179) suggesting this region may also have a key role to play in LT-tolerance (Table 3.6).

Interval mapping was also performed to identify QTLs that exceeded the significant LOD threshold score of 3.8 using MapQTL5 software. A QTL with a LOD score of 12.9 was revealed on chromosome 5A as indicated in Fig. 3.5 D. The analysis of all the genomic regions covered by the markers mapped in the Norstar X Cappelle-Desprez cross also indicated that the QTL present on chromosome 5A may contribute up to 80% towards the LT-tolerance trait (Fig.

Table 3.6 Chromosomal regions associated with LT- tolerance. K- value represents the Kruskal-wallis test statistic and *P*-value represents the significance of the associated marker with the LT-tolerance trait. Mean LT_{50} values of DH lines having Norstar or Cappelle-Desprez allele were used to determine which locus from the parents contributes to LT-tolerance in the Norstar X Cappelle-Desprez cross (Chodaparambil *et al.*, 2005; Båga *et al.*, 2006a).

Group	*Position (cM)*	*Locus (markers)*	*K*	*Significance (P)-value*	*Mean LT_{50} Norstar*	*Mean LT_{50} Cappelle*
1B	90	cfa2147_332	10.275	0.005	-17.6	-15.6
1B	108	gwm259_105	6.976	0.01	-17.6	-16.0
1B	112	E36M49_57	7.194	0.01	-17.6	-16.0
1B	118	gwm140_197	10.004	0.005	-17.6	-15.6
1D	10	wmc590_215	7.295	0.01	-18.2	-16.3
1D	10	barc229_(187C)	7.295	0.01	-18.2	-16.3
1D	19	barc169_122	7.274	0.01	-18.0	-16.2
4A	40	gwm397_188	6.978	0.01	-16.1	-17.7
4A	42	E36M61_(159C)	9.022	0.005	-16.0	-17.8
5A	43	E41M48_106	9.473	0.005	-18.2	-16.2
5A	46	barc180_199	9.061	0.005	-18.2	-16.2
5A	46	barc303_218	9.737	0.005	-18.3	-16.2
5A	46	gwm293_175	9.737	0.005	-18.3	-16.2
5A	46	barc186_210	9.737	0.005	-18.3	-16.2
5A	46	barc56_117	9.737	0.005	-18.3	-16.2
5A	46	gwm304_214	9.737	0.005	-18.3	-16.2
5A	46	barc122_236	9.737	0.005	-18.3	-16.2
5A	46	gwm129_130	9.737	0.005	-18.3	-16.2
5A	46	barc40_218	9.737	0.005	-18.3	-16.2
5A	49	barc186_200	9.429	0.005	-18.3	-16.3
5A	60	barc100_167	13.578	0.0005	-18.2	-15.7
5A	62	gwm156_289	13.045	0.0005	-18.1	-15.6
5A	65	gwm186_122	16.96	0.0001	-18.2	-15.5
5A	65	barc360_304	15.904	0.0001	-18.2	-15.5
5A	67	barc141_249	14.601	0.0005	-18.0	-15.5
5A	67	barc141_260	13.945	0.0005	-18.0	-15.5
5A	71	E36M61_803	23.128	0.0001	-18.4	-15.2
5A	78	wmc206_(212CM)	22.353	0.0001	-18.5	-15.4
5A	79	cfd2_326	26.854	0.0001	-18.6	-15.2
5A	79	barc197_193	26.854	0.0001	-18.6	-15.2
5A	79	gwm617_175	26.854	0.0001	-18.6	-15.2
5A	81	gwm639_(138C)	26.921	0.0001	-18.7	-15.2
5A	85	E40M60_(673C)	19.894	0.0001	-18.5	-15.6
5A	88	wmc327_183	15.932	0.0001	-18.3	-15.5
5A	98	gwm666_119	10.308	0.005	-18.0	-15.9

5A	98	wmc388_174	10.308	0.005	-18.0	-15.9
5A	98	E37M47_(346C)	10.308	0.005	-18.0	-15.9
5A	18	gwm410_271	7.416	0.01	-17.5	-15.7
5B	3	gwm499_152	6.852	0.01	-17.6	-16.1
5B	21	wmc75_205	8.483	0.005	-17.7	-16.0
5B	33	barc275_266	6.678	0.01	-17.6	-16.1
5B	40	barc232_218	7.323	0.01	-17.8	-16.2
5D	77	cfd8_158	11.654	0.001	-17.8	-15.8
5D	80	gwm583_165	9.938	0.005	-17.8	-16.0
5D	89	gwm174_179	11.918	0.001	-18.2	-16.0

3.5D). Genomic regions on chromosomes 1B (Fig. 3.5A), 3D (Fig. 3.5 B), 4A (Fig. 3.5C) and 5D (Fig. 3.5E) also showed regions that may contribute towards LT-tolerance but were not statistically significant and the putative QTLs identified on these chromosomes did not exceed the significant LOD threshold of 3.8 (Fig. 3.5). QTL analysis was not shown for all the chromosomes in Fig. 3.5 as they did not have statistically significant QTL. However, additional markers may be used to identify markers in these regions to identify markers that may exceed the significant LOD threshold.

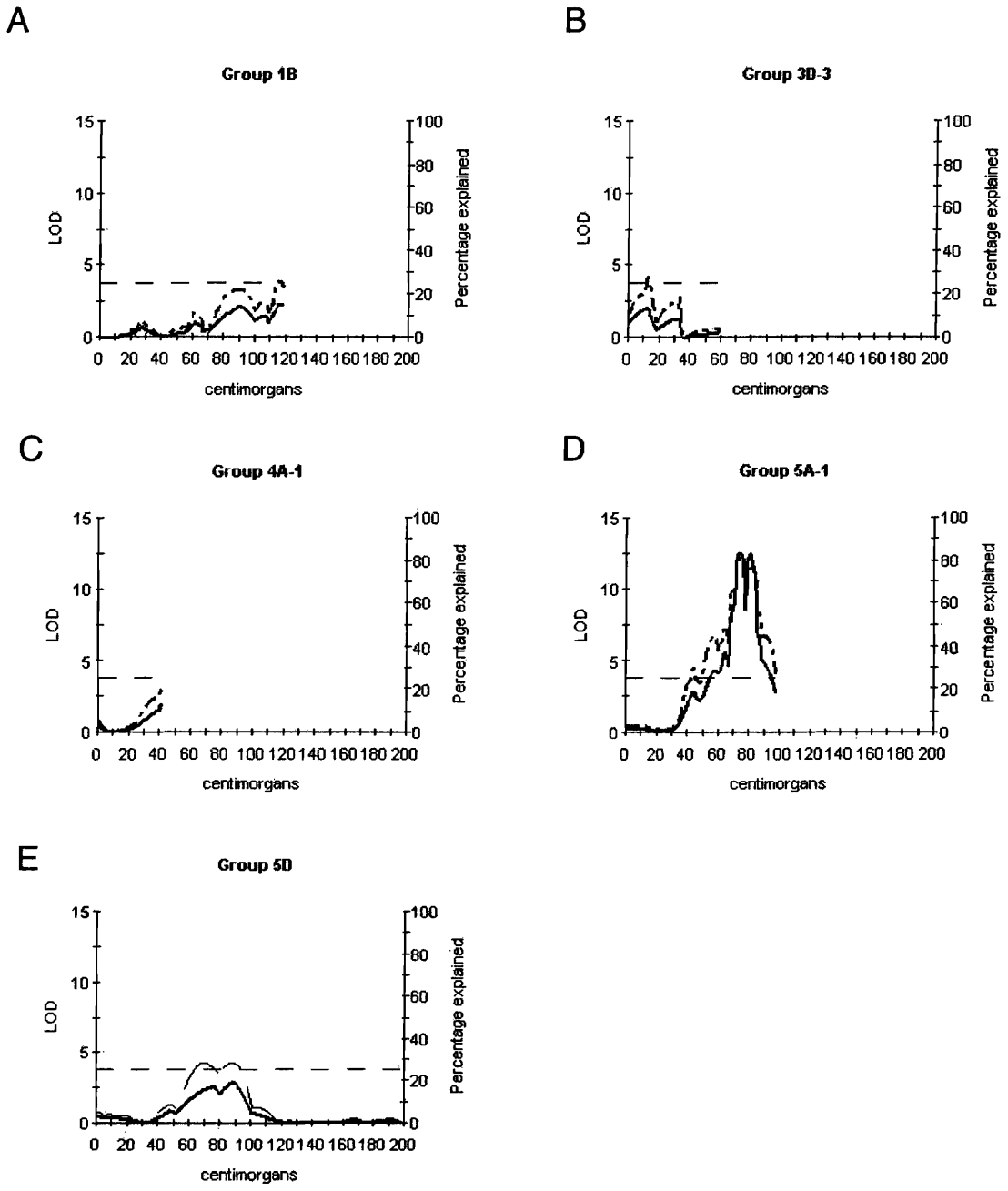


Figure 3.5 Interval mapping and identification of QTLs associated with LT-tolerance. Results are presented for choromosome groups 1B (**A**), 3D-3 (**B**), 4A-1 (**C**), 5A-1 (**D**), and 5D (**E**). The unbroken line indicates the QTL. The dashed lines overlapping the QTL represents the percentage of the LT-trait explained by the QTL. The horizontal dashed line indicates the genome-wide LOD threshold value (LOD = 3.8) in the Norstar X Cappelle-Desprez population.

3.4 Discussion

A DH population developed from the Norstar X Cappelle-Desprez cross was phenotyped for LT-tolerance and used in mapping to identify LT-tolerance associated regions (Båga *et al.*, 2006a, 2006b). In order to differentiate LT-tolerance and vernalization the cross was made using two winter wheat parents, Norstar and Cappelle-Desprez, having the same *vrn-1* region on chromosome 5A and differing only in LT-tolerance (Båga *et al.*, 2006a; 2006b). The DH population developed using the Norstar X Cappelle-Desprez cross was considered advantageous over developing other types of mapping populations as homozygous DH lines can be produced in a shorter time frame than developing RILs. Freeze tests have shown that none of the DH lines in the population significantly exceeded Norstar's LT-tolerance potential (Chodaparambil *et al.*, 2004, 2005; Båga *et al.*, 2006a, 2006b). This may be due to the lack of genetic variability in the Norstar X Cappelle-Desprez cross with regards to the ability to cold acclimate. The wheat cultivar Norstar has a Field Survival Index (FSI) of 514 and originated from a cross between wheat cultivars Alabaskaja (FSI 527) and Winalta (FSI 463) (Fowler, 1992). The wheat cultivar Ulianovkia, with an FSI index of 530, is amongst the highest in LT-tolerance potential. However, other winter cereals such as rye may have better genetic variability with regards to LT-tolerance as they have a FSI index of 735 which is far superior to the existing winter wheat cultivars and could be a source for identifying additional factors that are responsible for superior LT-tolerance (Fowler, 1992).

A linkage map of the Norstar X Cappelle-Desprez cross was assembled in an attempt to identify regions other than the *vrn-1* locus implicated in LT-tolerance. The Norstar X Cappelle-Desprez map spanned 2296 cM comprising 465 SSR markers and 107 AFLP markers distributed on 21 chromosomes. Recently, an integrative linkage map of winter wheat with a genetic length of 3086 cM was reported (Paillard *et al.*, 2003). In wheat, linkage maps have been reported with various genetic lengths: 3331 cM (Roder *et al.*, 1998), 3522 cM (Sourdille *et al.*, 2003), 2569 cM (Somers *et al.*, 2004), 3522 cM (Quarrie *et al.*, 2005), 3441 cM (Torada *et al.*, 2006). A total of 223 markers were mapped to the A genome, 245 markers were mapped to the B genome and 173 markers were mapped to the D genome. The results indicated that the D genome has a low level of polymorphism compared to the A and B genomes and is in agreement with earlier reports suggesting the low level of polymorphism is due to the monophyletic introduction of the D genome in hexaploid wheat (Lagudah *et al.*, 1991). The map positions of markers were in agreement with the SSR consensus map published by Somers *et al.* (2004) in hexaploid wheat. However, differences in marker order and discrepancies in size may be due to the low coverage of markers in certain segments of the genome where polymorphisms were not present, the low number of DH lines (40 in total), possible missing data points, and the genetic background of the cross. Chromosomes 1A, 1D, 2B, 3A, 3B, 3D, 4A, 4D, 5A, 5B, 6A, 6D, 7A, 7B and 7D had more than 1 linkage group due to the gaps in the region on account of low polymorphisms in the Norstar X Cappelle-Desprez cross and also due to the non-availability of markers in those

regions. However, utilizing the mapped SSR markers as anchor points in the Norstar X Cappelle-Desprez cross, a total of 197 AFLP markers were mapped on A genome - 70 markers, B genome - 88 markers and D genome - 30 markers. The AFLP markers mapped will help in identifying polymorphisms in regions where existing SSR markers do not reveal polymorphisms in other genetic backgrounds. Unique SSR markers mapped on the A genome (103 markers), B genome (110 markers) and D genome (103 markers) will also serve as additional information which will aid in the construction of further wheat linkage maps.

Single marker analysis on the Norstar X Cappelle-Desprez mapping population revealed putative QTLs on chromosomes 1B, 1D, 4A, 5A, 5B and 5D. Studies on vernalization sensitivity in wheat have indicated that the group 1 homologous chromosomes have a *vrn3* locus which influences the flowering time and may also contribute to the photoperiod response in wheat (Law and Worland, 1997). A similar study carried out to identify QTLs associated with LT-tolerance in a cross between winter wheat (Norstar) and spring wheat (Manitou) has also identified chromosome 1D in addition to chromosome 5A which contributes towards participating in LT-tolerance (Båga *et al.*, 2006b). The key difference in this study which uses a previously derived DH population from Norstar and Cappelle-Desprez (Båga *et al.*, 2006a, 2006b) was to neutralize the *vrn-1* region and possibly identify other chromosomal regions involved in LT-tolerance. The chromosome 1H in barley also has a vernalization locus (*vrn-H3*) and a photoperiod locus (*Ppd-2*) (Laurie *et al.*, 1995). Mapping for LT-tolerance traits in barley has implicated chromosome 1H in LT-tolerance response and a

putative QTL has been identified on this chromosome (Skinner *et al.*, 2005). Identification of markers associated with LT-tolerance on chromosomes 1B and 1D in the Norstar X Cappelle-Desprez cross indicates the possible role of these chromosomes in LT-tolerance response (Båga *et al.*, 2006b). However, more markers need to be mapped in this region to confirm the possibility of statistically significant QTLs in the Norstar X Cappelle-Desprez DH population. The homologous group 2 chromosomes are involved in photoperiod responses in wheat. In wheat, it is proposed that photoperiod sensitivity helps in maintaining and upregulating the LT-tolerance genes for longer duration under SD compared with LD environments (Mahfoozi *et al.*, 2000). However, there were no QTLs identified on the group 2 chromosomes using the Norstar X Cappelle-Desprez DH population. This may be due to the gaps present in the 2B chromosome in the Norstar X Cappelle-Desprez cross. The lack of allelic differences in the *Ppd* genes in the Norstar X Cappelle-Desprez cross may also be a factor for not identifying QTLs on the group 2 chromosomes.

The group 5 homologous chromosomes play a major role in LT-tolerance. LT-tolerance loci have been mapped to 5A (*Fr-A1*), 5B (*Fr-B1*) and 5D (*Fr-D1*) chromosomes (Galiba *et al.*, 1995; Snape *et al.*, 1997; Tóth *et al.*, 2003). Two loci have also been identified on chromosome 5A which are involved in regulating expression of the *cor14b* gene in LT-tolerant wheat. A LT-tolerance locus has also been located on the long arm of chromosome 5A about 30 cM proximal to the LT-tolerance locus *Fr-A1* in diploid wheat (*Triticum monococcum*) (Vagujfalvi *et al.*, 2003). Chromosome 5A also contains the regulatory genes

associated with the regulation of LT-tolerance associated genes *wcs120* and *wcor410* (Limin *et al.*, 1997; Danyluk *et al.*, 1998). The expression of several *CBF* genes present at the *Fr-A2* locus on chromosome 5A is also attributed to LT-tolerance in wheat (Vagujfalvi *et al.*, 2005). In the Norstar X Cappelle-Desprez DH population, a major LT-tolerance locus/loci was identified on chromosome 5A. The QTL identified on chromosome 5A in the Norstar X Cappelle-Desprez cross was confirmed by interval mapping and the peak of the QTL maps approximately to the same position as the LT-tolerance locus *Fr-A2*. The QTL mapped on the 5A chromosome indicates up to 80% of the LT-tolerance trait is conferred by this genomic region. Studies on LT-tolerance in other cereals, such as barley, have also revealed similar results, with the major LT-tolerance QTL being mapped to the 5H chromosome (Skinner *et al.*, 2005). The 5H, *Fr-H1* QTL is responsible for 62% of the phenotypic variation with regards to LT-tolerance (Skinner *et al.*, 2005). An additional *Fr-H2* locus in barley has been reported which comprises multiple *CBF* genes (Francia *et al.*, 2004; Skinner *et al.*, 2006). It is also possible that the QTL identified on chromosome 5A in wheat encodes for inducers of *CBF* genes, such as *ICE*, similar to that which has been observed in barley (Skinner *et al.*, 2005). Multiple QTL model analysis was not attempted on the Norstar X Cappelle-Deprez cross, as the mapping population used for QTL mapping was not random and the results obtained could be skewed. The use of additional DH lines for mapping would help in delineating the QTL on chromosome 5A further in the Norstar X Cappelle-Deprez cross.

4.0 C-REPEAT BINDING FACTORS (CBFs)

4.1 Introduction

C-Repeat Binding Factors (CBFs) or Dehydration Responsive Binding Elements (DREBs) are transcription factors that play an important role in the induction of *cor* genes. The CBFs regulate the expression of *cor* genes by binding to *cis* elements, known as C-repeat/Dehydration Responsive Elements (CRT/DREs), present in the promoters of *cor* genes. In Arabidopsis, 3 *CBF* genes (*CBF1/DREB1b, CBF2/DREB1c, CBF3/DREB1a*) have been identified and mapped to chromosome 4 (Gilmour *et al.*, 1998). The CBFs are conserved in both monocotyledonous and dicotyledonous plants and their transcripts are transiently up-regulated within 15 minutes of exposure of the plants to LT (Gilmour *et al.*, 1998; Jaglo *et al.*, 2001). *CBF* transcripts are induced during LT-exposure by a constitutive transcription factor, *inducer of CBF expression 1* (*ICE1*) (Chinnusamy *et al.*, 2003). Studies using *ice1* mutants in Arabidopsis have shown that these mutants block the activation of *CBF3* expression without affecting *CBF1* or *CBF2* expression when plants are exposed to LT (Chinnusamy *et al.*, 2003).

Orthologs of Arabidopsis *CBF* genes have also been identified in barley, rye and wheat. In barley, *HvCBF3* is a transcription factor that is induced by LT treatment. The *HvCBF3* transcription factor has been mapped to chromosome 5H in barley (Choi *et al.*, 2002). Similar to Arabidopsis, the barley *HvCBF3* gene is induced within 15 minutes in response to LT exposure and attains maximum expression in 2 hours, following which the level of expression declines and is

undetectable after 24 hours (Choi *et al.*, 2002). The orthologous chromosome in wheat, chromosome 5A has two major frost tolerant loci *Fr-A1* and *Fr-A2* that are involved in the regulation of *cor* genes such as *cor14b* (Vagujfalvi *et al.*, 2000; 2003). In barley, *HvCBF3* is an ortholog of *CBF3* locus in diploid wheat (*Triticum monococcum*) and has been mapped to the *Fr-A2* QTL (Vagujfalvi *et al.*, 2003). This locus affects the expression pattern of *cor14b*, indicating the involvement of the *CFB3* locus in the LT response (Vagujfalvi *et al.*, 2003). Recently, 20 *CBF* genes have been reported in barley which can be grouped into three multigene families (*HvCBF1, HvCBF3* and *HvCBF4*) based on their phylogeny (Skinner *et al.*, 2005; 2006). The 20 Arabidopsis *CBF* homologs identified in barley are present on the long-arm of chromosome 5H in two clusters and map to the *Fr-H1* and *Fr-H2* QTLs in barley (Skinner *et al.*, 2006; Tondelli *et al.*, 2006). The temporal and spatial expression of these genes varies in response to LT exposure, implying different subsets of the *HvCBF* genes may be expressed based on the nature of the LT-induced stress (Skinner *et al.*, 2005).

In winter wheat, genetic studies have implicated the *Vrn-A1/Fr-A1* locus to be involved with the LT-tolerance response (Sutka *et al.*, 1999). A second locus *Fr-A2* was also identified in diploid wheat (*Triticum monococcum*), 30 cM proximal to the *Vrn-A1/Fr-A1* locus involved in LT-tolerance (Vagujfalvi *et al.*, 2003). The *Fr-A2* locus also has the QTL associated with the *cor14b* and recently, 9 *CBF* genes have been identified and mapped to the *Fr-A2* region on chromosome 5A (Vagujfalvi *et al.*, 2003; Miller *et al.*, 2005). Studies in hexaploid wheat (*Triticum aestivum*) using chromosome 5A recombinant lines from hardy

and less-hardy wheat varieties have shown that 8 *CBF* genes mapped to the *Fr-A2* locus have a higher transcriptional level in a more hardy wheat cultivar (Vagujfalvi *et al.*, 2005). Although *CBF* genes present at the *Fr-A2* locus show different induction levels upon LT exposure, the specific *CBF* genes involved in determining a high level of LT-tolerance have not yet been identified (Vagujfalvi *et al.*, 2005). The genes *ICE1* and *FRY1* involved in the regulation of the *CBF* genes have been mapped in barley on chromosome 7H (Tondelli *et al.*, 2006). However, no QTL for LT-tolerance was identified on chromosome 7H. This suggests that the *CBF* genes may be better candidate genes for LT-tolerance in barley and other cereal species (Tondelli *et al.*, 2006).

The mapping results from the Norstar X Cappelle-Desprez cross presented in Chapter 3 also identified a major QTL corresponding to the *Fr-A2* locus in diploid wheat. To identify the possibility of *CBF* clones mapping to the *Fr-A2* locus in the Norstar X Cappelle-Desprez cross, a Norstar BAC library was screened. The fingerprinting of the putative clones may also provide an estimate of the total number of *CBF* genes that are present in this mapping population.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 BAC library

A previously generated BAC library of the winter wheat Norstar was utilized (Ratnayaka *et al.*, 2005). This library has in excess of 1.2 x 10^6 clones

stored at -80°C in 3,298, 384-well plates as glycerol stocks (Ratnayaka *et al.*, 2005). To facilitate screening, the entire library of 1.2 x 10^6 clones were double-spotted onto 69 high density 22.2 x 22.2 cm Hybond N^+ membrane filters (Amersham Biosciences, Piscataway, NJ) using a *Bio*Grid robot (BioRobotics, UK) at Agriculture and Agri-Food Canada (Saskatoon). Each high density Hybond N^+ membrane contained 18,432 clones double-spotted using the default 4 x 4 double offset pattern (Ratnayaka *et al.*, 2005).

4.2.1.2 CBF probe

A full length CBF fragment (950 bp), derived from a wheat cDNA clone (Genbank accession number: AB178167) was used as a probe to screen the Norstar BAC library. This cDNA clone was generously provided by Dr. Fathey Sarhan, Université du Québec à Montréal.

4.2.2 Methods

4.2.2.1 CBF probe preparation

4.2.2.1.1 Bacterial growth

Escherichia coli strain HB101 containing the plasmid pCMVsport (Invitrogen) with the 950 bp CBF insert was streaked on an LB plate (Appendix A) containing ampicillin (20 µg/mL) and grown at 37°C overnight. For plasmid isolation, a single colony was picked from the LB plate and grown overnight with agitation at 150 rpm in 150 mL of LB broth (Appendix A) with 20 µg/mL ampicillin.

4.2.2.1.2 Isolation of plasmid DNA

The overnight cultures of *E. coli* containing the plasmid were harvested by centrifugation at 4°C for 15 minutes at 6,000 x g in a Beckman J2-21 centrifuge (Fullerton, CA, USA). Plasmid extraction was performed by alkaline lysis using a Qiagen Plasmid Maxi kit (Qiagen cat no: 12163, ON, Canada) according to the manufacturers suggested directions. The DNA collected was precipitated with 0.7 volumes of 100% isopropanol and centrifuged at 15,000 x g for 30 minutes at 4°C. The subsequent DNA pellet was washed with 10 mL of cold 70% (v/v) ethanol, air dried for 10 minutes and dissolved in 150 µL of TE buffer (Appendix A).

4.2.2.1.3 Restriction digestion of DNA probe

The probe fragment was excised from the plasmid pCMVsport using the restriction enzymes *Eco*RI and *Hin*dIII. The digestion reaction contained the isolated plasmid pCMVsport (20 µg), 10 µL of *EcoR*I buffer (Appendix A), 2 µL *EcoR*I (40 U), 2 µL *Hind*III (40 U) and sterile H_2O to a final volume of a 100 µL. The reaction was incubated at 37°C for 2 hours.

4.2.2.1.4 Separation and purification of DNA probe

An aliquot (35 µL) of the digestion reaction was mixed with loading dye (Appendix A) and loaded in a 1% (w/v) agarose gel containing 20 µg of ethidium bromide. Electrophoresis occurred at 106 V constant voltage in a horizontal gel apparatus (Gibco) using TAE as a running buffer. The released CBF fragment

was visualized under UV light using a gel documentation apparatus (Chemi-Doc XRS, Bio-Rad) and subsequently purified from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturers suggested directions. The probe DNA was quantified using UV spectrophotometry (Beckman coulter DU800, Fullerton, CA, USA).

4.2.2.1.5 Labelling of DNA probe

The purified CBF probe fragment (25 ng) was labelled using ^{32}-P dCTP (Amersham, ON, Canada) using a Rediprime random Primers DNA Labelling System (Invitrogen, Cat.No: 18187-013) according to the manufacturers suggested directions. To remove the unincorporated nucleotides, the probe was purified through a Sephadex-25 NAP-5 column (Pharmacia Biotech Cat. No: 52-2074-00). The NAP-5 column was equilibrated using TE buffer prior to use. The labelled CBF probe reaction mixture (50 µL), was made up to a total volume of 500 µL by adding TE buffer (Appendix A). This was applied to the center of the NAP-5 column and allowed to enter the gel bed completely. The probe was eluted with 1 mL of TE buffer and specific activity was determined using a Geiger counter (TBM 3-S, TA Technical associates, CA, USA).

4.2.2.2 BAC library primary screening

Each high density Hybond-N$^+$ membrane used for screening comprised 18,432 clones which were double-spotted using the default 4 x 4 double offset pattern (Ratnayaka *et al.*, 2005). The membranes were wetted with 5X SSPE

and pre-hybridized at 65°C for 1 hour in Church buffer (Appendix A) (Church and Gilbert, 1984). Membrane hybridizations were performed overnight in a hybridization oven (Biometra OV3, Goettingen, Germany) set at 65°C using ^{32}P-labeled CBF probe (1 x 10^8 dpm/mL) and 15 mL of Church buffer. The membranes were washed twice in 1X SSC with 0.1% (w/v) SDS for 20 minutes at 65°C. The washed filters were air-dried and exposed to Kodax BioMax maximum sensitivity film (Kodak, New Haven, CT, USA) for 24 to 48 hours at -80°C depending on the signal intensities and developed using an automated X-ray processor (AFP Imaging, NY, USA).

4.2.2.3 Identification and secondary screening of clones

The positive CBF clones were identified using the double-spotted hybridization signal obtained from the high-density filter. A grid representing the pattern in which the colonies were printed on the membrane was placed over the autoradiogram and the address of the clones was determined based on the gridding pattern of the *Bio*Grid Robot (BioRobotics, UK). The addresses of the positive CBF clones were deciphered and the clones were picked from master plates (384 well plates) using a sterile toothpick. Ninety-six well microtiter plates containing LB media with chloramphenicol (12.5 µg/mL) were inoculated with the selected colonies. The colonies were grown overnight at 37°C in an incubator (Max Q 5000, VWR, ON, Canada) and the cultures were manually gridded on a Hybond N^+ membrane. The filter was processed by treating for 7 minutes in a denaturation solution (Appendix A) followed by soaking the membranes in

neutralization solution (Appendix A) for 7 minutes. The filter was then wetted with 0.4 M NaOH for 20 minutes followed by soaking in 5X SSPE (Appendix A) for 7 minutes. Cross-linking was performed with a Stratalinker UV crosslinker (Stratagene, La Jolla, CA). The filter was allowed to dry at room temperature. The filter was re-probed with the CBF fragment as describe in section 4.2.2.2 to confirm that the positive clones picked from the master plates were from the correct address.

4.2.3 Fingerprinting of putative clones

4.2.3.1 Bacterial growth

The *E. coli* cells containing the positive BAC clones identified with the CBF probe were grown from glycerol stocks as described in section 4.2.2.3 except that the bacteria was inoculated into 100 mL of LB media containing chloramphenicol (12.5 µg/mL) in a conical flask and grown overnight in a shaker set at 37°C and 150 rpm.

4.2.3.2 Isolation of BAC DNA

The overnight cultures of *E. coli* containing the BAC were harvested by centrifugation at 4°C for 10 minutes at 5,000 x g in a centrifuge (Sorvall). The BAC DNA was extracted using a PhasePrep BAC DNA Kit (Sigma, Product code NA0100) according to the manufacturers suggested instructions. The DNA pellet obtained was washed with 500 µL of room temperature 70% (v/v) ethanol and centrifuged at 15,000 x g for 5 minutes. The supernatant was removed with a

pipette tip and the DNA pellet was air-dried and dissolved in 15 µL of TE buffer. The DNA was quantified using UV spectrophotometry (Beckman coulter DU800, Fullerton, CA, USA).

4.2.3.3 Restriction digestion fingerprinting

BAC DNA from each clone was digested using the restriction enzyme *Hind*III. The digestion reaction contained the BAC DNA (500 ng), 3 µL of *Hind*III buffer (Appendix A), 1 µL *Hind*III (20 U) and sterile H_2O to a final volume of a 30 µL. The reaction was incubated at 37°C overnight. The entire digestion reaction (30 µL) was mixed with loading dye and loaded in a 1% (w/v) agarose gel containing 20 µg of ethidium bromide. Electrophoresis occurred at 40 V constant voltage for 12 hours in a horizontal gel apparatus (Gibco) using 1X TBE as a running buffer. The digested DNA fragments were visualized under UV light using a gel documentation apparatus (Chemi-Doc XRS, Bio-Rad). Quantity One software (Bio-Rad) was used to determine the sizes of the digestion fragments.

The gels were soaked for 20 minutes each in depurination solution, denaturation solution and neutralization solution (Appendix A). Gels were blotted to Hybond-N^+ membrane in 20X SSC for 1 hour using a PosiBlot apparatus (Stratagene) or overnight by standard Southern transfer (Southern, 1975). Membranes were crosslinked with UV crosslinker (Stratalinker, Stratagene) and probed with the CBF probe as described in section 4.2.2.2 to identify the fragments hybridizing to the CBF sequence.

4.3 Results

4.3.1 BAC library screening

The Norstar BAC library which was used for identifying CBF clones was constructed previously and contains an average insert size of 75 kb (Ratnayaka *et al.*, 2005). This library has a theoretical coverage of 5.5X and a validated coverage of 4.4X haploid genome equivalents and there is a 99.6% probability of finding a desired DNA sequence (Ratnayaka *et al.*, 2005). In total, 62 high density filters comprising 1,142,784 clones were screened to identify positive CBF clones using a full-length wheat cDNA CBF fragment as a probe. Clones were identified as positive CBF clones when a duplicated signal pattern as gridded by the *Bio*Grid robot (BioRobotics, UK) was observed as shown in the representative Fig. 4.1A.

The primary screening of 62 filters identified 163 putative CBF clones. The addresses of the clones on the master plates were identified for all 163 clones and are presented in Appendix E. All of the 163 clones were manually picked from the master library plates and grown in 96 well microtiter plates. In order to validate the results and to check if the correct colonies were chosen from the master plates, all the 163 clones were gridded to a Hybond N^+ membrane and reprobed with the CBF probe. There were large variations in signal strength of the positive CBF clones and only 4 clones did not hybridize to the CBF probe in the secondary screening (Fig. 4.1B).

4.3.2 *Hind*III fingerprinting of the putative CBF BAC clones

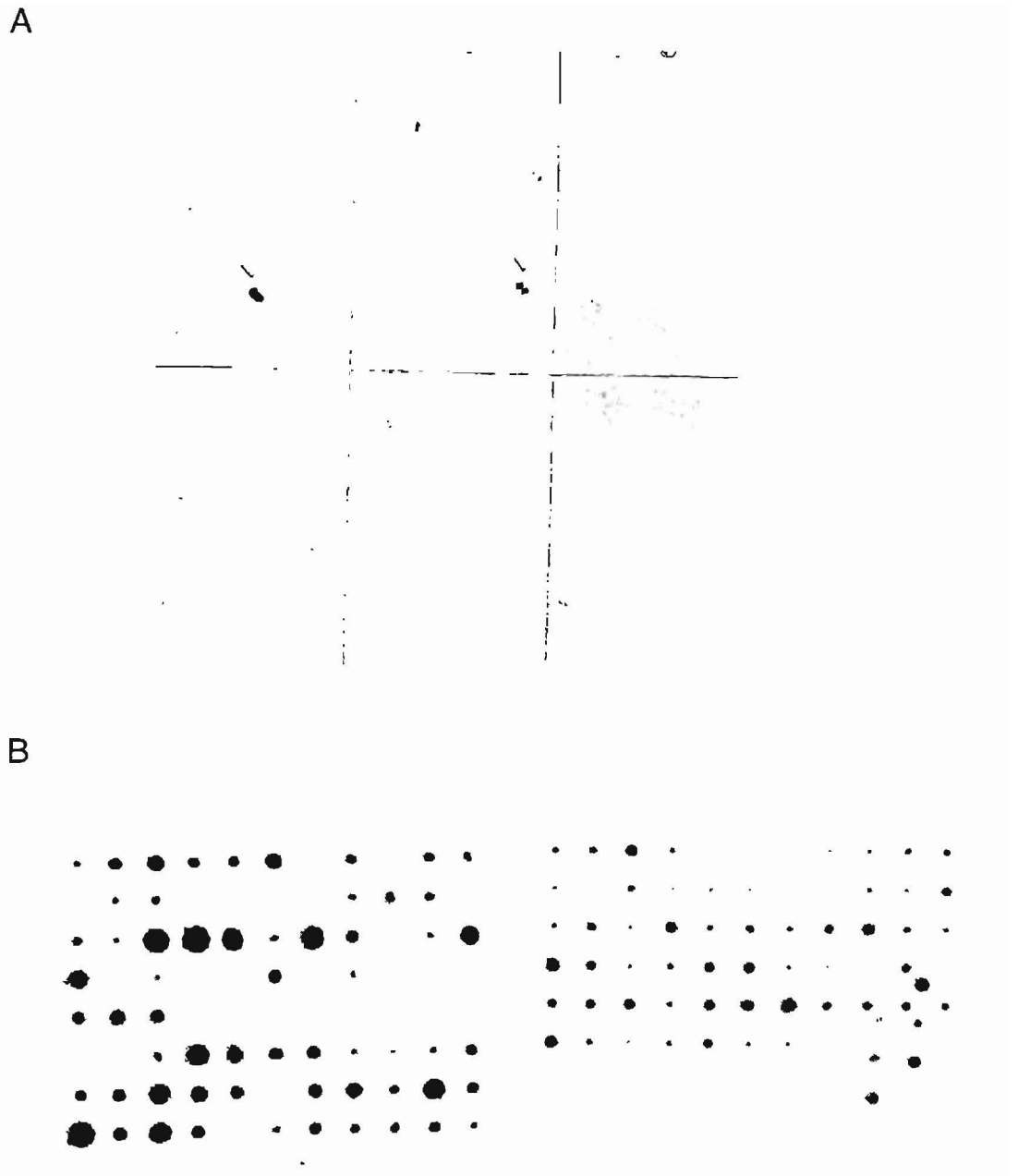


Figure 4.1 BAC library screening of for CBF positive clones. (**A**) Primary screening of high density filters carrying 18, 432 double-spotted clones. Positive CBF clones are identified by a duplicated signal pattern after hybridization to ^{32}P-labelled CBF probe as indicated by the arrows. A representative autoradiograph is shown. (**B**) Secondary screening of the CBF positive clones.

Eighty-one clones with the strongest hybridization signals from the secondary screen were selected out of the 163 clones for large-scale plasmid preparation followed by *Hind*III fingerprinting. A representative gel is shown in Fig. 4.2A. The digested DNA was transferred to membranes which were subsequently probed with the CBF probe to identify the fragments hybridizing to the CBF sequence. A representative blot is shown in Fig. 4.2B.

*Hind*III fingerprinting of the 81 clones yielded a total of 187 fragments that hybridized with the CBF probe and a total of 539 non-hybridizing fragments. Seven clones did not have any CBF hybridizing fragments and were not included in further analyses. The CBF probe hybridizing fragments ranged from 1 kb to 33.7 kb, while the non-CBF hybridizing fragments ranged from 0.6 kb to 33 kb. Based on the *Hind*III fingerprints, the largest clone was 1-F12 with a size of 114.9 kb and the smallest clone was 1-B11 with a size of 23.1 kb. The average size of the CBF positive clones identified in this study was 73.63 kb. The fragment sizes of the CBF probe hybridizing bands from the different CBF positive clones are presented in Table 4.1.

An analysis of all the clones for the number of unique *Hind*III restriction fragments indicated that 54 such unique CBF-hybridization fragments were present (Table 4.1). The designation of the clones in Table 4.1 is based on the address of the clone in the master plate, for example, clone 1-C5 is a clone from the first 96-well master plate in the position C-5. A strategy was devised to group the tentative CBF clones based on the *Hind*III fingerprint pattern. The CBF clones with similar CBF hybridizing fragment were placed in one group. Similar

non-CBF hybridizing fragments were also taken into account while grouping the clones in a specific group. Clones with similar non-CBF hybridizing fragment were placed in the same orientation if the clones shared other similar CBF-hybridizing fragments else, the clones were positioned in the opposite orientation in the putative contig assembly (Appendix F). Based on the *Hind*III fingerprint pattern, the clones were grouped as shown in (Table 4.2).

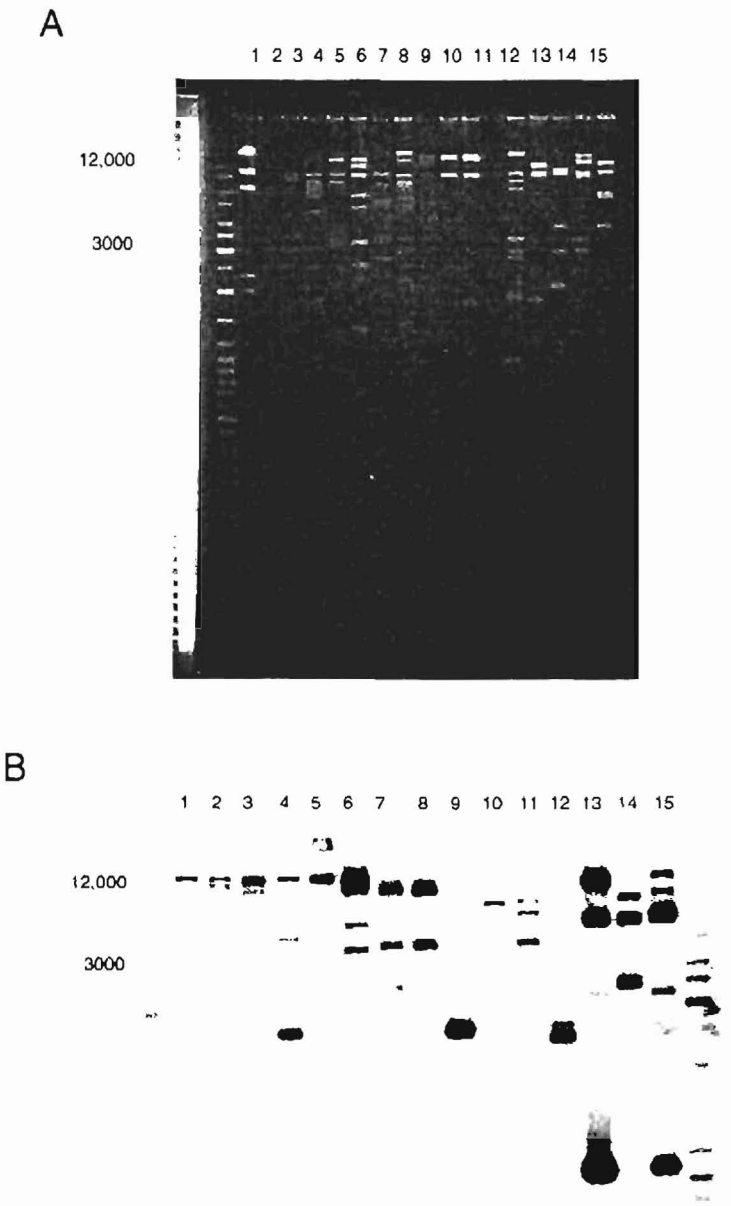


Figure 4.2 *Hin*dIII fingerprinting of putative CBF clones. Select clones identified from the secondary screen were subjected to *Hin*dIII restriction digestion (lanes 1 through 15) (**A**) and subsequently hybridized to a ^{32}P-labelled CBF probe (**B**). Representative results for *Hin*dIII restriction profiles and CBF hybridizing fragments are shown.

Table 4.1 Size of the *Hin*dIII restriction digested fragments from putative CBF clones. Sizes were determined for fragments hybridizing to a ^{32}P-labelled CBF probe.

Clone	*size (kb)*		*Clone*	*size (kb)*		*Clone*	*size (kb)*
1-C5	1		2-C10	6.2		1-G5	12.5
1-H5	1		1-C5	6.5		1-H11	12.6
2-E7	1		2-A10	6.5		1-C5	12.9
1-H2	1.1		1-F5	6.6		2-A11	13
1-H4	1.1		2-E6	6.6		1-F6	13.2
1-H8	2		2-E7	6.7		1-G4	13.2
2-A3	2		2-E10	6.7		1-H2	13.3
2-E2	2		1-A3	6.8		2-E1	13.4
1-A9	2.3		1-G12	6.8		1-F12	13.8
1-A6	2.4		1-C5	6.9		2-E11	13.8
1-H1	2.4		1-H5	6.9		1-G1	14
2-D5	2.4		1-C6	7		1-D2	15.2
1-G2	2.6		2-D5	7		1-A7	15.5
1-G8	2.6		2-D2	7.1		2-A10	15.5
1-H1	2.6		1-A11	7.2		2-C4	15.8
1-A1	2.9		2-E6	7.2		2-F1	15.9
1-C2	2.9		2-E8	7.2		1-G1	16.1
1-H4	3		1-A3	7.3		1-G10	16.3
2-E1	3		2-C4	7.3		2-E3	16.5
2-E11	3.1		1-E2	7.4		1-E4	16.7
1-C5	3.2		2-C10	7.6		2-D5	17.1
1-H5	3.2		2-E12	7.7		2-E7	17.1
1-A1	3.4		2-D1	7.8		2-D10	17.4
1-G5	3.4		1-G10	7.9		1-B4	17.5
1-H3	3.4		2-C6	7.9		1-F12	17.6
2-E11	3.5		2-C8	7.9		1-G4	17.6
1-E3	4.4		1-F6	8		1-D2	17.7
1-A9	4.7		1-C5	8.1		1-C8	18.2
1-D1	4.7		2-A11	8.2		1-G2	18.2
1-G4	4.8		2-E10	8.2		1-C9	18.4
2-E11	4.8		1-E3	8.4		1-H10	18.6
1-G5	4.9		1-H3	8.4		1-A12	18.9
1-G6	4.9		1-C2	8.5		1-B1	19
1-G12	4.9		1-G12	8.5		1-C5	19
2-E1	4.9		1-F5	8.6		2-D2	19.1
1-E4	5		1-H4	8.6		1-H8	19.4
2-D2	5		1-C5	8.8		1-H11	19.5
1-G9	5		1-H5	8.8		2-E9	19.7
2-D5	5.1		2-C6	9		1-G3	19.9
1-G2	5.2		2-C8	9		2-E10	20.1

1-G12	5.2		2-E11	9		2-E6	20.4
1-E2	5.3		2-E12	9.1		2-E11	20.6
1-F8	5.3		1-G9	9.1		1-H4	20.8
1-C4	5.4		1-C1	9.2		1-G4	21
1-C12	5.4		1-E2	9.2		1-G1	21.3
1-F7	5.4		2-A11	9.2		2-E2	21.7
1-C9	5.5		2-E2	9.2		1-A11	22.1
2-D1	5.5		1-G11	9.4		2-B11	22.3
1-C8	5.6		1-G12	9.5		1-F12	22.5
2-C6	5.6		2-D5	9.7		1-F11	22.8
2-C8	5.6		2-E5	9.9		2-E10	23
1-G11	5.6		1-C9	10		2-E1	24.2
1-C4	5.7		1-C12	10.1		1-H9	24.4
1-F6	5.7		2-C6	10.1		2-E9	24.9
1-C8	5.8		2-C8	10.2		2-E11	25.2
1-C9	5.8		1-C8	10.3		2-E7	25.4
1-C12	5.8		2-D1	10.5		2-D10	25.8
2-E11	5.8		2-D2	10.7		1-H5	26.4
2-E12	5.8		2-F1	11		2-D1	27.5
1-G4	6		1-B11	11.5		2-C8	29.5
2-F1	6		1-D2	11.5		2-C4	31.2
1-H2	6.1		1-D1	11.8		2-C2	31.8
1-H3	6.2		1-G6	11.8		2-A11	33
1-H4	6.2		2-C2	11.9		2-A10	33.7

Table 4.2 Grouping of putative CBF clones

Grouping	*Clones*
Group 1[A]	1-H5, 2-E7
Group 2[B]	1-C5[C], 1-H2[C], 1-H4
Group 3[D]	1-H8[E], 2-A3[E], 2-E2[E], 1-H11
Group 4[F]	1-H1, 1-G2
Group 5[G]	1-A6, 2-D5
Group 6[H]	1-D1[I], 1-G4[I], 1-A9[J], 2-E11[J]
Group 7[K]	1-G5[L], 1-G6[L], 2-E1[M], 1-G12[M]
Group 8[N]	1-G1, 2-D2
Group 9[O]	1-D1[P], 1-C4[P], 1-C12[Q]
Group 10[R]	1-E2[S], 1-F8[S], 1-F7[T]
Group 11[U]	1-C9[V], 1-C8[V], 2-C6[W], 2-C8[W], 1-G11
Group 12[X]	1-H3, 2-C10
Group 13[Y]	2-E6, 2-E10
Group 14[Z]	2-E8, 2-C4
Group 15[AA]	1-A1, 1-C2, 1-E3, 1-E4, 1-F6, 2-E12, 2-F1, 2-A10, 1-F5, 1-C6, 1-A11, 1-A3, 1-G10
Group 16[AB]	2-A11, 1-C1, 2-E5, 1-B11, 1-D2, 2-C2, 1-A7, 1-G1, 2-E3, 2-D10[AC], 1-B4[AC], 1-F12, 1-H10, 1-A12, 1-B1, 2-E9, 1-G3, 2-B11, 1-F11, 1-H9

[A]share a 1.0 kb CBF hybridizing fragment
[B]share a 1.1 kb CBF hybridizing
[C]share 2.1 kb and 3.3 kb non-CBF hybridizing fragments
[D] share a 2.1 kb CBF hybridizing fragment
[E]share a 3.9 non-hybridizing fragment
[F]share a 2.7 kb CBF hybridizing fragment and a 2.1 kb, 2.3 kb, 2.9 kb and 3.1 kb non-hybridizing fragment
[G]share a 2.5 kb CBF hybridizing fragment and 3.2 and 3.6 kb non-hybridizing fragments
[H]share a 4.8 kb CBF hybridizing fragment was grouped together.
[I]shared 1.1 kb, 1.2 kb, 1.6 kb, 2.4 kb, 2.8 kb and 5.5 kb non-CBF hybridizing fragments
[J]share a 2.0 kb non-CBF hybridizing fragment
[K]share a 4.9 kb CBF hybridizing fragment.
[L]share a 2.1 kb and 2.3 kb non-CBF hybridizing fragments
[M]share a 3.5 non-CBF hybridizing fragment
[N]share a 5.1 kb CBF hybridizing fragment
[O]share a 5.5 kb CBF hybridizing fragment and a 3.8 kb non-CBF hybridizing fragment
[P]share a 8.9 kb non-CBF hybridizing fragment
[Q]share a 5.8 kb CBF hybridizing fragment

[R]share a similar 5.4 kb CBF hybridizing band and a 1.6 kb non-CBF hybridizing fragment
[S]share a 2.1 kb non-CBF hybridizing fragment
[T]share share 1.8 kb, 2.5 kb and 2.8 kb non-CBF hybridizing fragments
[U]share a 5.6 kb CBF hybridizing fragment.
[V]share 5. 9 kb, 10 kb, 18 kb CBF hybridizing fragments
[W]share 8 kb, 9 kb, 10 kb CBF hybridizing fragments and 2.2 kb, 2.8 kb and 4.6 kb non-CBF hybridizing fragments
[X]share a 6.2 kb CBF hybridizing fragment
[Y]share a 6.7 kb CBF hybridizing fragment
[Z]share a 7.3 kb CBF hybridizing fragment.
[AA]comprises 13 unique clones based on CBF hybridizing fragments
[AB]comprises 20 clones with CBF hybridizing fragment size greater than 8 kb, 18 of which contain a unique CBF hybridizing fragment
[AC]share a similar 17.5 kb CBF hybridizing fragment

4.4 Discussion

CBF transcriptional factors are transiently and rapidly expressed upon exposure of plants to LT (Thomashow, 1998). The CBF proteins are members of the AP2/EREBP protein family but possess additional sequences surrounding the AP2/EREBP domain and are conserved in plants such as wheat, rye, brassica and tomato (Jaglo *et al.*, 2001). However, the number of *CBF* genes present in plants and their temporal and spatial expression vary (Jaglo *et al.*, 2001). Studies by Jaglo *et al.* (2001) revealed that in barley, *HvCBF3* expression is maximum at 2 hours following low-temperature exposure, declining steadily thereafter. In contrast, wheat and rye *CBF* genes were continuously expressed from 30 minutes to 24 hours upon exposure of the plants to LT (Jaglo *et al.*, 2001).

A BAC library of wheat was used to identify positive CBF clones. Although the BAC library has coverage of 5.5 X, the average insert size of the library is only 75 kb (Ratnayaka *et al.*, 2005). This represents a potential

limitation due to the large number of clones which must be screened and also complicates physical mapping and contig assemblies. Fingerprinting analysis using *Hin*dIII on the putative CBF clones and hybridization using the CBF probe has identified the CBF hybridizing fragments and assisted in a tentative grouping of the clones. Based on the fingerprint pattern of the clones, it may be reasoned that the hexaploid Norstar genome may contain at least 54 CBFs, considering that there may be *CBF* gene paralogs for each of the three genomes A, B and D. This is in agreement with the results obtained in barley where 20 CBFs have been reported, and in diploid wheat where 11 CBF clones have been reported (Skinner *et al.*, 2006; Miller *et al.*, 2005). The mapping results obtained using the Norstar X Cappelle-Desprez cross is also in agreement with results obtained by Skinner *et al.*, (2005) in barley and Miller *et al.*, (2005) in wheat implicating the *Fr-2* region for low-temperature tolerance and for the presence of *CBF* genes mapping to that region. However, the specific *CBF* gene(s) that are involved or that may contribute towards low-temperature tolerance have not been identified yet in Norstar. The *Fr-2* region in wheat and barley also encode the transcriptional activators involved in the regulation of *cor14b* gene involved in LT-tolerance (Vagujfalvi *et al.*, 2003; Francia *et al.*, 2004). Studies on the *Fr-2* locus in hexaploid wheat have indicated that certain *CBF* genes (*CBF1A, CBF1C and CBF7*) have higher transcript levels in the hardy genotypes compared to their less-hardy hardy counterparts (Vagujfalvi *et al.*, 2005). However no specific CBF gene(s) have been implicated that may help explain the difference in phenotypic

variation associated with low-temperature tolerance at this locus (Vagujfalvi *et al.*, 2005).

While CBF transcription factors may play a major role in the ability of plants to develop LT-tolerance, it should also be noted that the ability of plants to acquire LT-tolerance depends on a complex interaction of various factors. In Arabidopsis, the *CBF* genes are themselves regulated by *ICE1* which is constitutively expressed. Furthermore, mutation at the *ICE1* locus blocks the induction of *CBF3* gene expression (Chinnusamy *et al.*, 2003). The signaling response and the cold sensing mechanisms which activate ICE1 in response to LT are not well known but are essential to better understand the LT signaling process. The recent identification of ZAT12 transcription factors that increase LT-tolerance upon constitutive expression as well as downregulate the expression of certain CBFs indicate it could be an additional cold response pathway (Vogel *et al.*, 2005). However, it needs to be ascertained if the same ZAT12 cold responsive pathway exists in wheat and other cereals.

In Arabidopsis, three *CBF* genes have been identified and are present in tandem on chromosome 4 (Gilmour *et al.*, 1998). *CBF* genes and their map positions have also been recently reported in barley as well as diploid and hexaploid wheat (Vagujfalvi *et al.*, 2003; 2005; Skinner *et al.*, 2006). In barley, 20 *CBF* genes have been identified and map to the 5H-L chromosome in 2 clusters coinciding with the *Fr-H1* and *Fr-H2* QTLs implicated in LT responses (Skinner *et al.*, 2006; Tondelli *et al.*, 2006). The characterization of a *CBF* gene family in barley has also provided information on the expression and complexity

of the *CBF* genes. The *CBF* genes have been phylogenetically characterized and grouped into three subgroups: *HvCBF1*, *HvCBF3* and *HvCBF4* based on the *CBF* subfamily signature motif that flanks the conserved AP2 sequences (Skinner *et al.*, 2005). All of the monocot *CBF* genes lack introns and can be grouped into one of the three barley CBF subgroups indicating the conservation of *CBF* genes in cereals (Skinner *et al.*, 2005). Over-expression of barley *CBFs* in Arabidopsis have revealed that *HvCBF3* and *HvCBF6* subgroup members can induce the expression of *cor* genes at 20°C, whereas the *HvCBF4* subgroup can activate expression of reporter genes only at 2°C, suggesting LT is essential for this group of transcription factors to be active (Skinner *et al.*, 2005). Similar results have also been obtained in wheat (*TaCBF1*) and rye (*ScCBF22*, *ScCBF24* and *ScCBF31*) *HvCBF4* subgroup members (Skinner *et al.*, 2005). In *Triticum monococcum*, a cluster of 11 *CBF* transcription factors have been mapped at the *Fr-A^{m}2* LT-tolerance locus (Miller *et al.*, 2005). Characterization of the *CBF* genes identified in wheat has revealed close similarities with *CBF* genes identified in barley, indicating that the *CBF* genes existed before the divergence between wheat and rice about 50 million years ago (Miller *et al.*, 2005). The *CBF* genes identified in *Triticum monococcum* are divided as subgroup A (similar to the barley *HvCBF3* subgroup), subgroup B and subgroup C which have been mapped to the chromosome 7A^m and share close similarities with the barley *CBF* gene subgroups.

Based on the observations in diploid wheat and barley, it is probable that the *CBF* genes in hexaploid wheat may also be present in clusters.

It may be hypothesized, based on the *Hind*III restriction fingerprinting, that at least 54 *CBF* genes are present in the hexaploid wheat Norstar. Sequencing the CBF fragments and assembly of the clones identified in this study will help in discovery of such possible clusters as well as overlapping regions and duplications. Based on the linkage map of Norstar X Cappelle-Desprez cross, it will be possible to assign a *CBF* gene or a cluster of *CBF* genes to different regions of the chromosome and also identify the possible *CBF* gene(s) involved in LT-tolerance.

5.0 MATURE EMBRYO REGENERATION

5.1 Introduction

A suitable *in vitro* regeneration system is an essential aspect of plant transformation studies which ensures a whole plant can be regenerated from tissue, organs or cells isolated from the explant. Whole plant regeneration from explant is possible due to the plasticity and totipotency exhibited by the plant cell which allows the plant to initiate tissue or organs from different cell types and also maintain the total genetic potential of the parent plant (Dodds and Roberts, 1995). However, in order to achieve a relatively good regeneration potential, *in vitro* culture conditions are crucial. An *in vitro* culture media is usually composed of 1) a carbon source 2) macro- and micro-nutrients and 3) vitamins, amino acids and plant growth regulators (Dodds and Roberts, 1995). The *in vitro* culture media can be solid or liquid based on the need. Different classes of plant growth regulators have been used in *in vitro* culture media: auxins, cytokinins, gibberellins and abscisic acid (Smith, 2000). While auxins promote cell division and cell growth, cytokinins are known to promote only cell division (Smith, 2000). The plant growth regulator gibberellin regulates cell elongation and abscisic acid inhibits cell division. The concentration and the ratios of auxins and cytokinins used in the culture media have a profound effect on the explant used for regeneration. While a low auxin to high cytokinin ratio induces shoot formation, a high auxin to low cytokinin ratio leads to root formation, and an intermediate ratio of auxin and cytokinins produces callus, which are usually comprised of unspecialized parenchyma cells (Smith, 2000).

Different types of explants have been used for *in vitro* regeneration: protoplasts, apical meristems, microspore and embryos (Smith, 2000). Protoplasts are usually obtained by enzymatic isolation or mechanical isolation from leaf cells by removing the cell wall. Apical meristems obtained from shoot tips also serve as a good source of explant tissue. Microspore culture can also be used to produce haploid tissue, which may be treated with colchicine to induce chromosome doubling. Immature and mature embryos also serve as an excellent source of explant to generate callus cultures or somatic embryos. Somatic embryos can be produced indirectly via a callus phase, or directly from a small group of cells without the intervening callus phase by altering the concentration of auxin in the media (Dodds and Roberts, 1995).

Tissue culture and regeneration of plants is crucial for successful crop improvement and functional genomics programs. The prerequisites required for obtaining transformed plants include 1) a suitable explant, 2) an efficient *in vitro* regeneration system and 3) a competent transformation system. With the current advances in cereal genomics, the need for the development of an efficient regeneration system to study genetically transformed cereals has become more imperative. Tissue culture and regeneration in wheat is not only dependent on genotype, but also on the explant source and the media used for regeneration. In addition, different genotypes may have specific media requirements in order to obtain optimum results (Sears and Deckard, 1982).

In wheat, various explants such as scutellum, immature embryos and mature embryos have been successfully used for regeneration (Ahloowalia,

1982; Ahuja *et al.*, 1982; Ozias-Atkins and Vasil, 1983; Maddock *et al.*, 1983; He *et al.*, 1988). In addition, immature inflorescences and immature leaves have also been successfully used as explants for the regeneration of fertile plants in four Canadian wheat cultivars (Caswell *et al.*, 2000). Scutellum is used as a regular source of explant in the regeneration of wheat, and an average of 16 plantlets can be obtained from one cultured scutellum after the initiation of somatic embryogenesis (Bommineni and Jauhar, 1996). Immature embryos have also been used routinely in regeneration because of the high frequency of callus induction obtained when using this source as the explant (Redway *et al.*, 1990). However, to use scutella and immature embryos as explants, the donor plants have to be grown throughout the year to maintain a supply of donor explant material. When these tissues are used as an explant source, the stage of harvesting is critical to ensure good regeneration potential and hence many additional factors must be considered in order to obtain good results. Scutellar tissues have also been used to develop somatic embryos in wheat and used for genetic transformation using microprojectile bombardment to obtain herbicide-resistant, self-fertile, transgenic plants (Nehra *et al.*, 1994). Recently, a study examining the regeneration of scutellum tissue in four commercial durum wheat cultivars showed that there is a significant cultivar X media interaction upon inclusion of various plant growth regulators in the growth culture media (Satyavathi *et al.*, 2004).

The problem of harvesting immature embryos and scutellum at an appropriate stage can be circumvented by the use of mature embryos as the

explant source. Mature embryos have been used successfully as an explant in the regeneration of wheat (Özgen *et al.*, 1998; Delporte *et al.*, 2001; Zale *et al.*, 2004). Mature embryos are a better source of explants not only due to their easy availability, but also because of the physiological state of the mature embryo (Delporte *et al.*, 2001). Studies examining the use of mature embryos as an explant demonstrated a 90% callus induction rate, and a total of 25 to 30 plants were obtained per 100 embryos (Delporte *et al.*, 2001). Mature embryos of hexaploid and durum wheat have been successfully used for genetic transformation by particle bombardment (Patnaik and Khurana, 2003).

To date, plant regeneration from explant tissues in wheat has occurred via a callus phase. Plant growth regulators including 2,4-dichlorophenoxyacetic acid (2-4-D), indole acetic acid (IAA), napthaleneacetic acid (NAA), 6-benzylaminopurine (6-BAP) and various cytokinins have been successfully used for embryogenic callus induction and plant regeneration from various explants of wheat. Thidiazuron (TDZ) (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea), is a plant growth regulator with high cytokinin activity which promotes the growth of cytokinin-dependent callus cultures (Mok *et al.*, 1982). Compared to the endogenous cytokinins, TDZ is active at lower concentrations compared to other cytokinins and is less susceptible to degradation (Mok *et al.*, 1987). In wheat, when a comparison was made between several kinetins and various auxin sources such as 2,4-D, Dicamba (3,6-dichloro-o-anisic acid) and NAA using immature embryos for the purpose of *in vitro* regeneration, the use of TDZ in the media obtained the best results (Shan *et al.*, 2000).

Experiments were performed to test the regeneration potential of mature embryos in different wheat cultivars and genotypes using TDZ and BAP as a source of cytokinin. TDZ was specifically chosen as one of the plant growth regulators due to the positive impact of TDZ on the regeneration potential in both dicot and monocot plants.

5.2 Materials and Methods

5.2.1 Plant materials

Seeds of winter wheat (*Triticum aestivum* CDC Clair, CDC Osprey and CDC Ptarmigan) were obtained from the Crop Development Center, University of Saskatchewan (Saskatoon, Saskatchewan). Seeds of spring wheat (*Triticum aestivum* AC Nanda) and durum wheat (*Triticum turgidum* AC Plenty) were procured from Agriculture and Agri-Food Canada (Saskatoon, Saskatchewan).

5.2.2 Culture media compositions

All growth culture media used in this study for shoot regeneration contained the macro- and micro-elements as described by Murashige and Skoog (1962), as well as 3% (w/v) maltose, 1 g/L enzymatic casein hydrolysate, B5 vitamins (Gamborg *et al.*, 1968), 0.7 g/L L-proline, 5 μM copper sulphate and 0.8% (w/v) phytagar (Sigma). The cytokinins used in this study, BAP (6-benzylaminopurine) and TDZ (Thidiazuron), were included in the media at different concentrations. The media was prepared with sterile de-ionized water

and autoclaved. The media was allowed to cool and poured into 9-cm Petri-plates in a laminar flow hood. Solidified plates were stored at room temperature.

Culture media for rooting contained the macro- and micro-elements as described by Murashige and Skoog (1962) with 1 mg/L indolebutyric acid and 0.8% (w/v) phytagar. After preparation as described above, the media was transferred to 15 cm long x 2.5 cm diameter screw-cap tubes in 15 mL aliquots.

5.2.3 Sterilization of seeds and embryo dissection

Surface-sterilization of seeds was done by shaking the seeds in commercial bleach (Javex, 5.25% (v/v) sodium hypochlorite) for 45 minutes on an orbital shaker (G10, New Brunswick scientific, Canada) set at 150 rpm, followed by rinsing in sterile de-ionized water. This was repeated 3 times. The surface-sterilized seeds were transferred to Petri-plates with sterile de-ionized water and imbibed overnight at 4°C. Mature embryos were isolated from the imbibed seeds with a sharp scalpel which was used to dislodge the mature embryo from its attachment to the scutellum. The dissected embryos were immediately transferred to various culture media as described in section 5.2.2. A dissection microscope was used to aid in the dissection of the embryo. The area around the microscope was wiped periodically with 70% (v/v) ethanol to maintain sterile conditions. The entire procedure involving dissection of embryos and transfer to culture media was performed in a laminar flow hood to reduce contamination and to maintain a sterile environment. A total of 12,000 embryos, 300 explants for each culture media per cultivar, were used in this study.

5.2.4 Culturing conditions

The isolated embryos were incubated on media with different concentrations of cytokinins (Table 5.1) in the dark at 24°C for 3 weeks. After 3 weeks, the cultures were exposed to low-light conditions (150 µmol m^{-2} s^{-1}) for 1 week and subsequently sub-cultured with fresh media with the same concentration of cytokinin and incubated at 24°C under high-light conditions (350 µmol m^{-2} s^{-1}) with a 16/8 hour light/dark cycle. Sub-culturing was performed every 3 weeks after trimming the shoots to 1 cm in length. Sub-culturing occurred twice during the study. Regeneration frequency (or shoot induction per embryo) is defined as the total number of shoots produced from one embryo at the end of 10 weeks. The number of shoots regenerated from embryos was counted manually by separating the shoots from the shoot clump.

In total, 1,680 regenerants (5 genotypes X 8 media compositions X 42 regenerants) were selected to be transferred to the rooting media. The cultures were incubated at 24°C under high-light conditions (350 µmol m^{-2} s^{-1}) with a 16/8 hour light/dark cycle for 1 month to establish roots. Plants with established roots, were transferred to plastic pots containing moist Redi-earth (W.R. Grace & Co. Ontario, Canada) and covered with transparent plastic cups to maintain humidity. Only the winter wheat varieties established roots and hence the plants required vernalization. This occurred in a controlled environment chamber for 8 weeks at 4°C with a 16/8 hour light/dark cycle and a PPFD of 350 µmol m^{-2} s^{-1}. At this time the plants were returned to greenhouse conditions as described in section 3.2.1 until the plants matured and set seeds.

5.2.5 Statistical analyses

Each cultivar cultured on each medium was termed as one experimental unit. The mean number of shoots derived from 20 embryos that were grown on a 9-cm diameter Petri-dish made up one experimental unit. The experimental design used for the shoot regeneration study was a 5 cultivar X 8 culture medium factorial in a 15 replicate randomized complete block design. The general linear model procedure was used for analysis of variance of the data using the SAS software package, Version 8.0 (SAS Institute Inc., Cary, NC). LSD was determined using experimental errors determined from the ANOVA table.

5.3 Results

Mature embryos of wheat genotypes: CDC Osprey, CDC Clair, CDC Ptarmigan, AC Nanda and AC Plenty were isolated from imbibed seeds. A total of 12,000 mature embryos were excised and used to identify the best media requirements for the individual wheat genotypes. Eight different types of media with different concentrations of cytokinins were used to study the regeneration responses of the 5 wheat genotypes as shown in table 5.1. ANOVA analyses for all the genotypes examined indicated that the different media compositions used in this study varied and were statistically significant amongst them at $P \leq 0.01$.

Table 5.1 Concentrations of cytokinins used in shooting culture media.

Culture media	*Cytokinin*	
	TDZ (mg/ L)	*BAP (mg/ L)*
MBT1	1.0	0.0
MBT2	2.0	0.0
MBR3A	0.0	1.0
MBR4A	0.0	2.0
T1B1	1.0	1.0
T1B2	1.0	2.0
T2B1	2.0	1.0
T2B2	2.0	2.0

5.3.1 Shoot formation in winter wheat

5.3.1.1 CDC Osprey

Winter wheat CDC Osprey responded well in the medium T2B1 (average of 20 shoots per explant), T1B1 (average of 19 shoots per explant) and T2B2 (average of 20 shoots per explant) (Fig. 5.1A) which were considered the best media for shoot production in CDC Osprey based on the LSD test ($P \leq 0.05$) (Appendix G). When TDZ (1 mg/L) was present in combination with BAP (2 mg/L) (T1B2) the number of shoots produced per explant averaged 18 (Fig. 5.1A). The study also shows that the presence of only TDZ in the medium as in MBT1 (1 mg/L) or MBT2 (2 mg/L), produced a lower number of shoots in the explants with an average of 15 and 17 shoots respectively (Fig. 5.1A). The absence of TDZ from the medium produced an average of two shoots as observed when using the medium MBR4A and MBR3A (Fig 5.1A). The shoot formation in cultivar CDC Osprey when grown on medium MBT2 and MBR4A is shown in Figs. 5.1B and 5.1C.

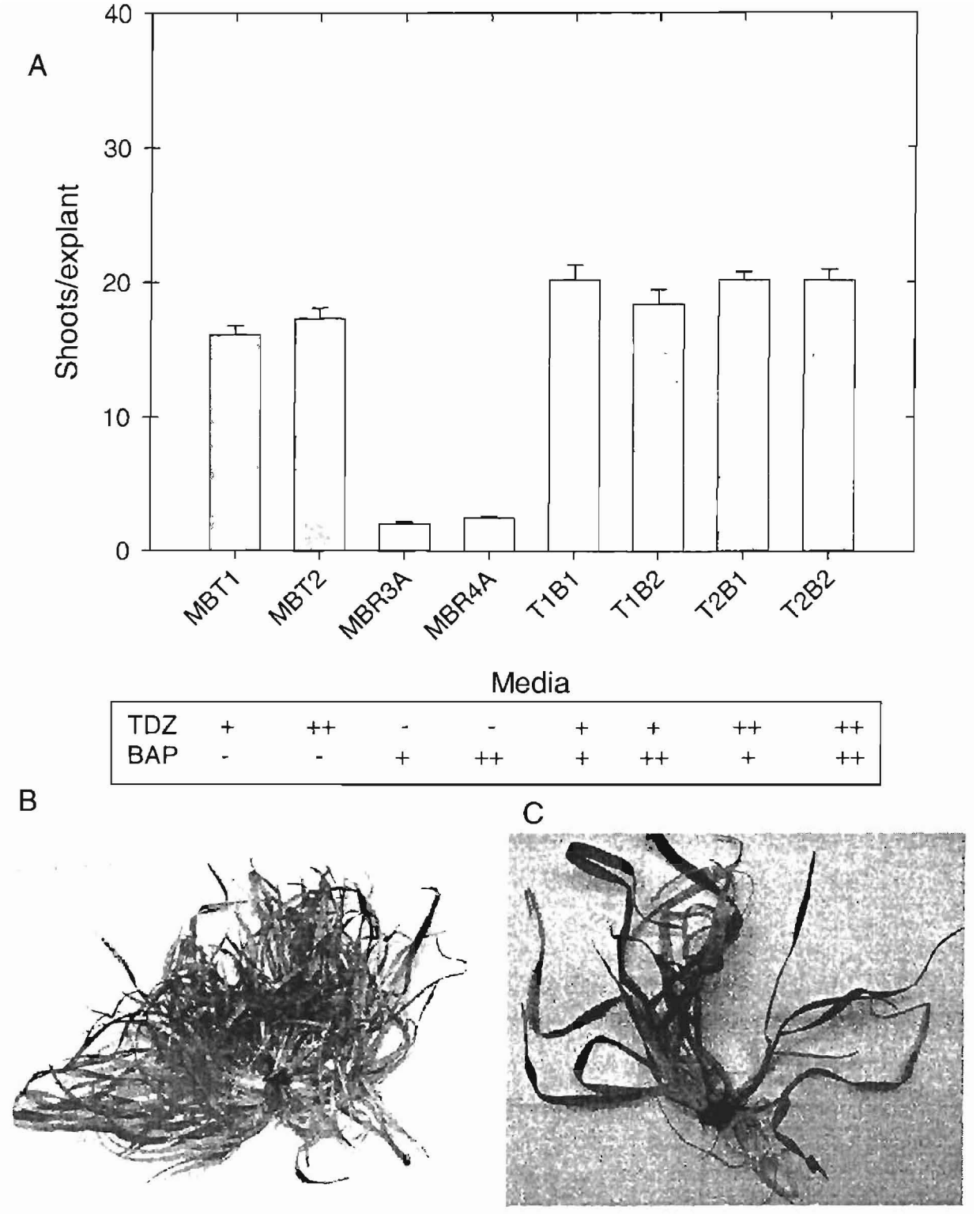
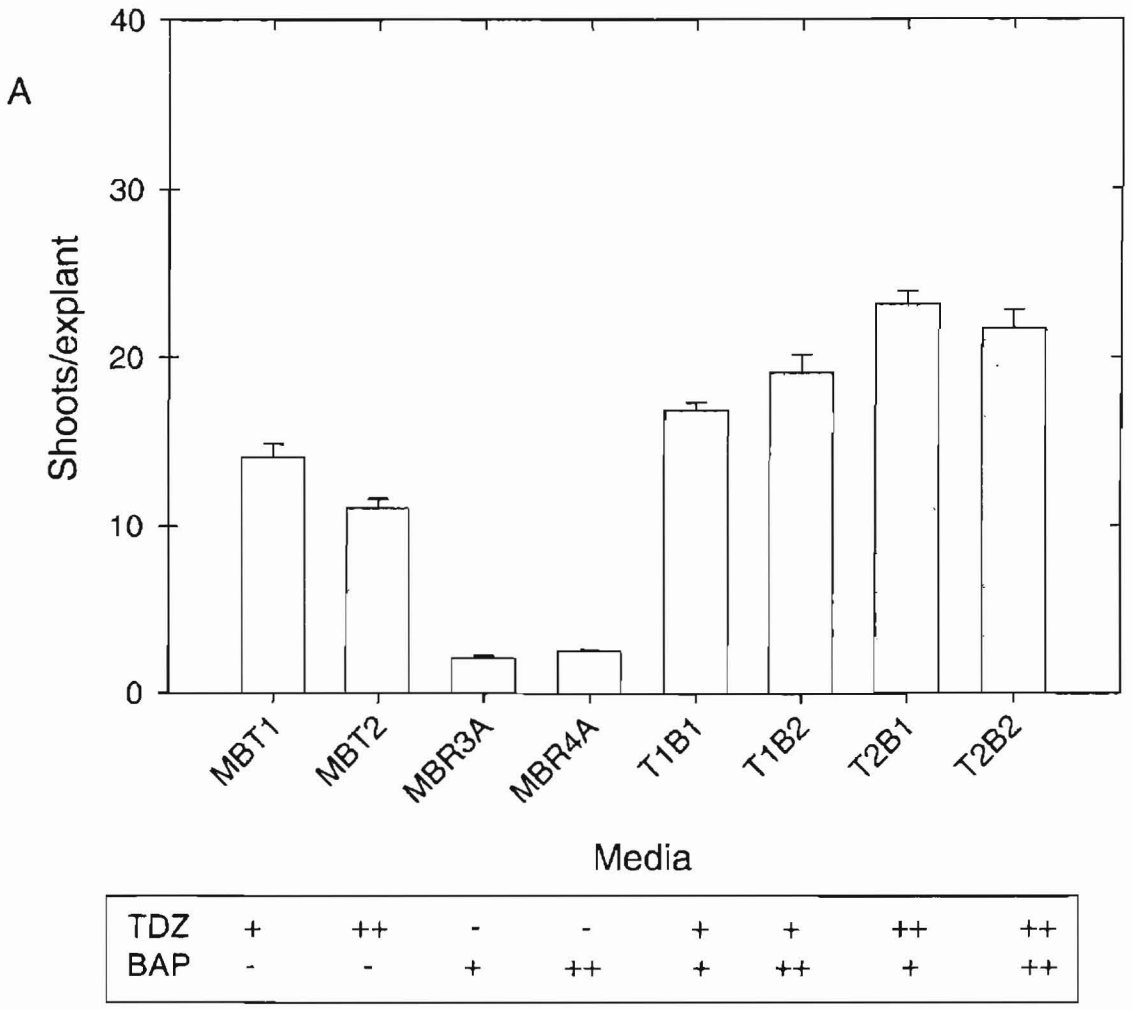


Figure 5.1 Shoot formation in winter wheat (*T. aestivum* CDC Osprey). Number of shoots per explant (**A**). Values represent means ± SE, $n = 15$. Symbol (+) represents 1 mg/L of TDZ or BAP, (++) represents 2 mg/L of TDZ or BAP, (-) represents TDZ or BAP was not added to the medium. A representative photograph for explants cultured on media MBT2 (**B**) and MBR4A (**C**) is shown.

5.3.1.2 CDC Clair

Winter wheat CDC Clair responded well in the media T2B1 and T2B2, producing an average of 22 and 21 shoots per explant respectively (Fig. 5.2A) and were considered the most statistically significant media based on the LSD test ($P \leq 0.05$) (Appendix G). The results indicate that increasing the concentration of BAP in the media (T2B2) does not necessarily increase the number of shoots produced by the explants compared to the media T2B1 (Fig. 5.2A). However, when the concentration of TDZ was decreased to 1 mg/L (T1B1 and T1B2) in combination with 1 mg/L and 2 mg/L of BAP the number of shoots regenerated per explant significantly decreased to an average of 16 and 19 shoots respectively (Fig. 5.2A). When explants were grown on MBR3A and MBR4A medium, less than 5 shoots were produced per explant. When media with TDZ was used without supplementing with BAP (MBT1 and MBT2) the number of shoots produced per explant declined further to an average of 13 and 11 shoots respectively (Fig. 5.2A). This suggests that the inclusion of BAP in the media has a positive effect on shoot regeneration (Fig 5.2A). However, the complete absence of TDZ in the media caused a further decline of shoot formation to an average of 2 shoots per explant, confirming the stimulatory effect of TDZ at a concentration of 1 mg/L in the medium(Fig. 5.2A). The shoot formation in cultivar CDC Clair when grown on medium T1B2 and MBR4A is shown in Figs. 5.2B and 5.2C.



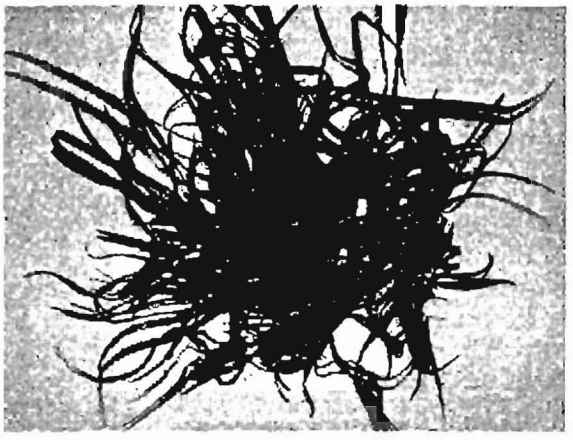


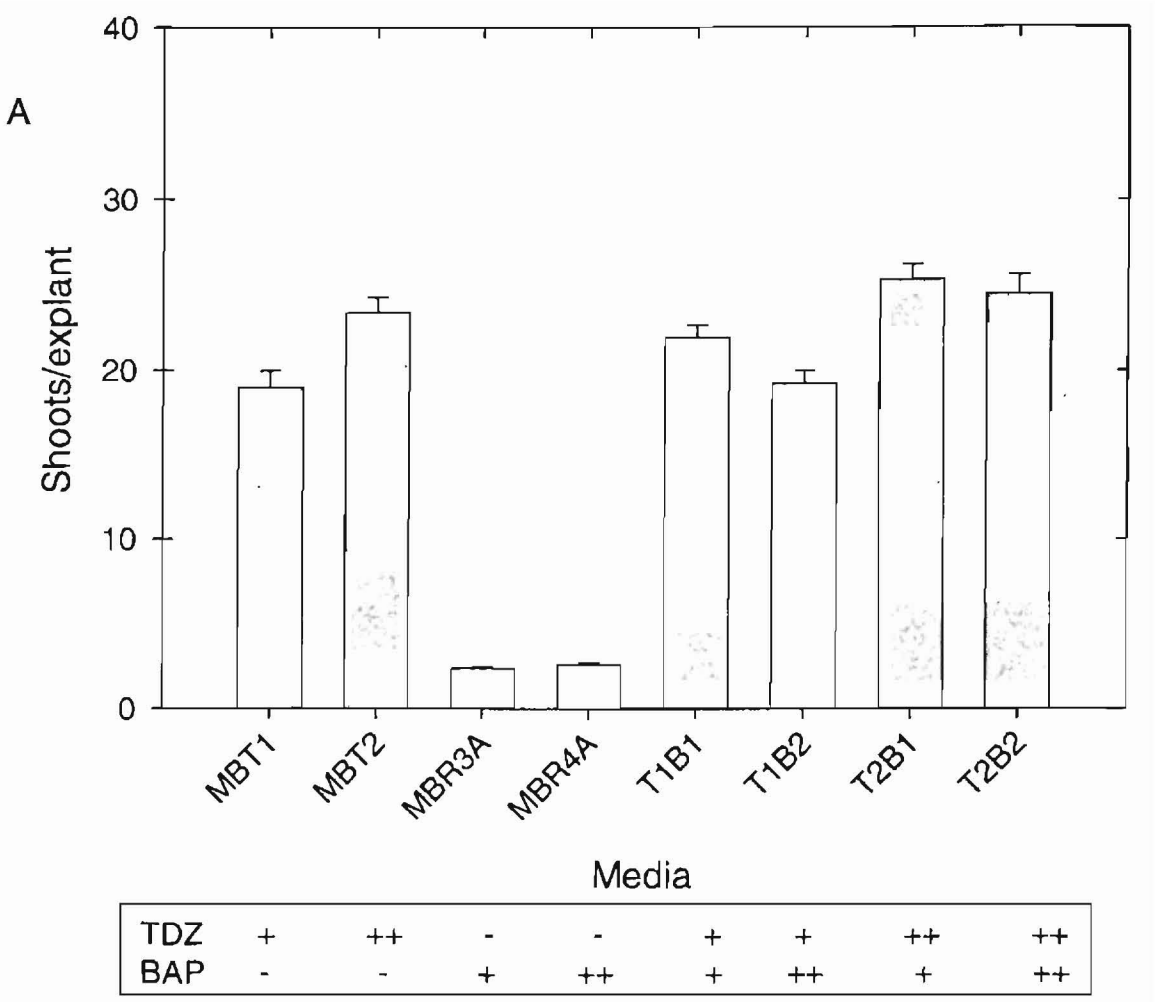
Figure 5.2 Shoot formation in winter wheat (*T. aestivum* CDC Clair). Number of shoots per explant (**A**). Values represent means ± SE, $n = 15$. Symbol (+) represents 1 mg/L of TDZ or BAP, (++) represents 2 mg/L of TDZ or BAP, (-) represents TDZ or BAP was not added to the medium. A representative photograph for explants cultured on media T1B2 (**A**) and MBR4A (**B**) is shown.

5.3.1.3 CDC Ptarmigan

Winter wheat CDC Ptarmigan responded well in all media compositions containing TDZ: T2B1 (average of 25 shoots per explant), T2B2 (average of 24 shoots per explant), MBT2 (average of 25 shoots per explant), T1B1 (average of 22 shoots per explant) and T1B2 (average of 19 shoots per explant) (Fig. 5.3A). However, media T2B1 and T2B2 were considered the most statistically significant based on the LSD test ($P \leq 0.05$) (Appendix G). When TDZ was excluded from the media as in MBR4A and MBR3A the number of shoots regenerated decreased to an average of 2 shoots per explant. This indicates the presence of TDZ in the media is necessary for increased shoot production (Fig. 5.3A). The shoot formation in cultivar CDC Ptarmigan when grown on medium T1B2 and MBR4A is shown in Figs. 5.3B and 5.3C.

5.3.2 Shoot formation in spring wheat

Spring wheat AC Nanda was the only cultivar used in this study which demonstrated that one media, T2B1, with an average of 27 shoots per explant (Fig. 5.4A), was the most statistically significant medium based on the LSD test ($P \leq 0.05$) (Appendix G). A combination of TDZ and BAP used in the media T2B2, T1B2 and T1B1 did not show a significant increase in the number of shoots produced per explant (Fig. 5.4A). However, when BAP was not present in the media (MBT2 and MBT1), the number of shoots produced per explant reduced to an average of 21 and 17 shoots, respectively (Fig. 5.4A). The absence of TDZ in



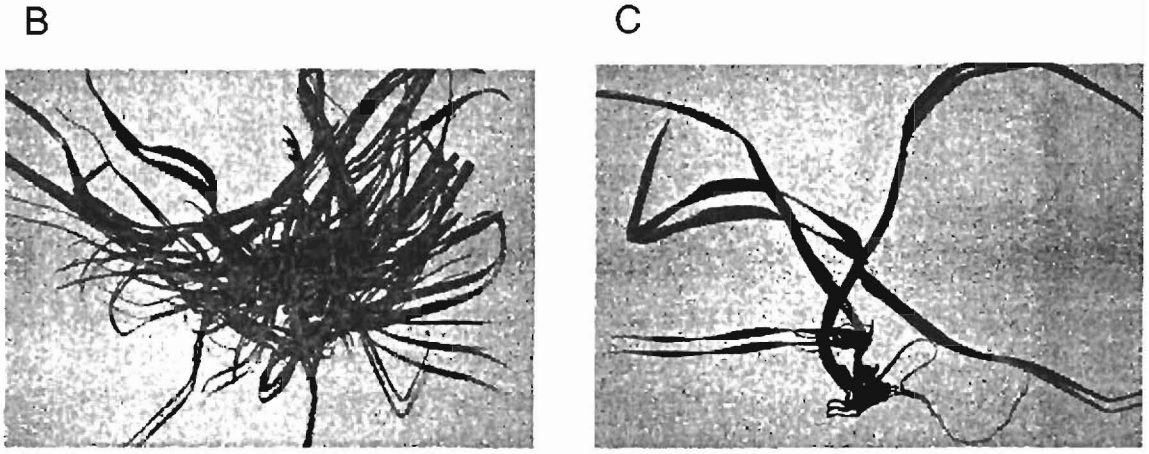
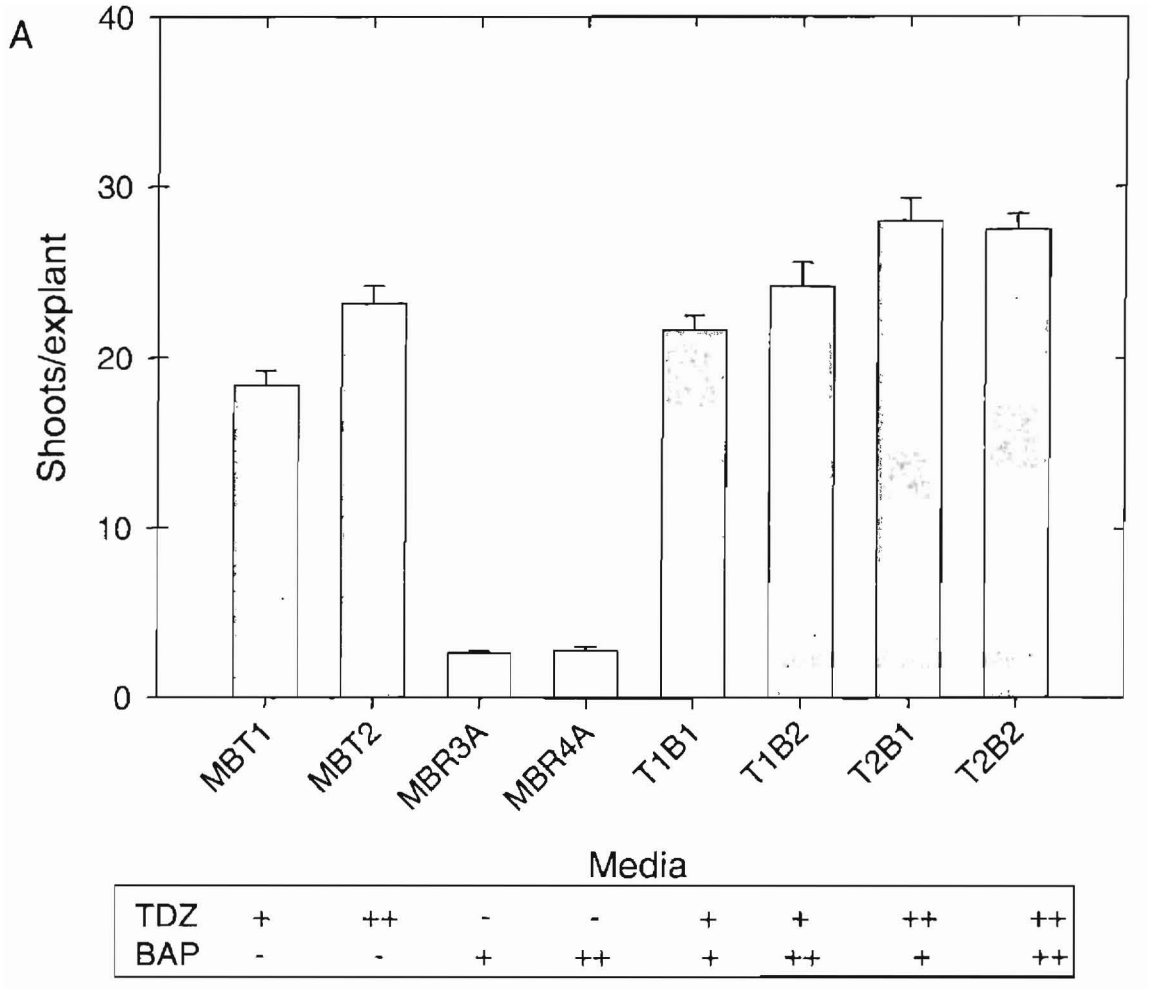


Figure 5.3 Shoot formation in winter wheat (*T. aestivum* CDC Ptarmigan). Number of shoots per explant (**A**). Values represent means ± SE, $n = 15$. Symbol (+) represents1 mg/L of TDZ or BAP, (++) represents 2 mg/L of TDZ or BAP, (-) represents TDZ or BAP was not added to the medium. A representative photograph for explants cultured on media T1B2 (**B**) and MBR4A (**C**) is shown.



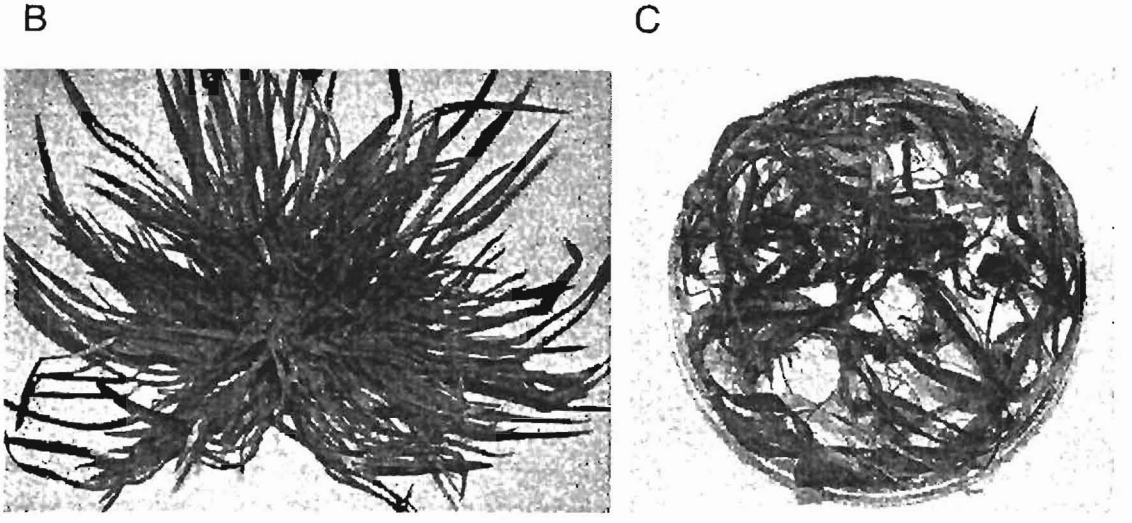


Figure 5.4 Shoot formation in spring wheat (*T. aestivum* AC Nanda). Number of shoots per explant (**A**). Values represent means ± SE, $n = 15$. Symbol (+) represents1 mg/L of TDZ or BAP, (++) represents 2 mg/L of TDZ or BAP, (-) represents TDZ or BAP was not added to the medium. A representative photograph for explants cultured on media T1B2 (**B**) and MBR3A (**C**) is shown.

the media further reduced the average number of shoots produced to 2 as in the case of media MBR4A and MBR3A (Fig. 5.4A). The multiple shoot formation in cultivar AC Nanda when grown on T1B2 media as well as growth on MBR3A media is shown in Figs. 5.4B and 5.4C.

5.3.3 Shoot formation in durum wheat

In the durum wheat cultivar AC Plenty, T1B1 and T1B2 were considered the most statistically significant media for shoot formation with an average of 34 and 33 shoots respectively (Fig. 5.5A) based on the LSD test ($P \leq 0.05$) (Appendix G). An increase in the concentration of TDZ in the medium (T2B2 and T2B1) did not significantly increase the shoot production with an average of 27 and 28 shoots per explant respectively (Fig. 5.5A). Increasing the concentration of TDZ in the media to 2 mg/L without BAP (MBT2) resulted in similar shoot production as in the case of T2B2 and T2B1 media (Fig. 5.5A). However, a decrease in the concentration of TDZ to 1 mg/L without BAP (MBT1) reduced the average number of shoots produced per explant to 28 (Fig. 5.5A). It was also noted that only durum wheat produced an average of five shoots per explant when cultured on the media MBR4A and produced the greatest number of shoots per explant amongst all the wheat genotypes used in this study using this media composition (Fig 5.5A). Media MBR3A formed the least amount of shoots with an average of 2 shoots per explant (Fig. 5.5A). Figs. 5.5B and 5.5C show the effect of media T1B2 and MBR3A on the cultivar AC Plenty.

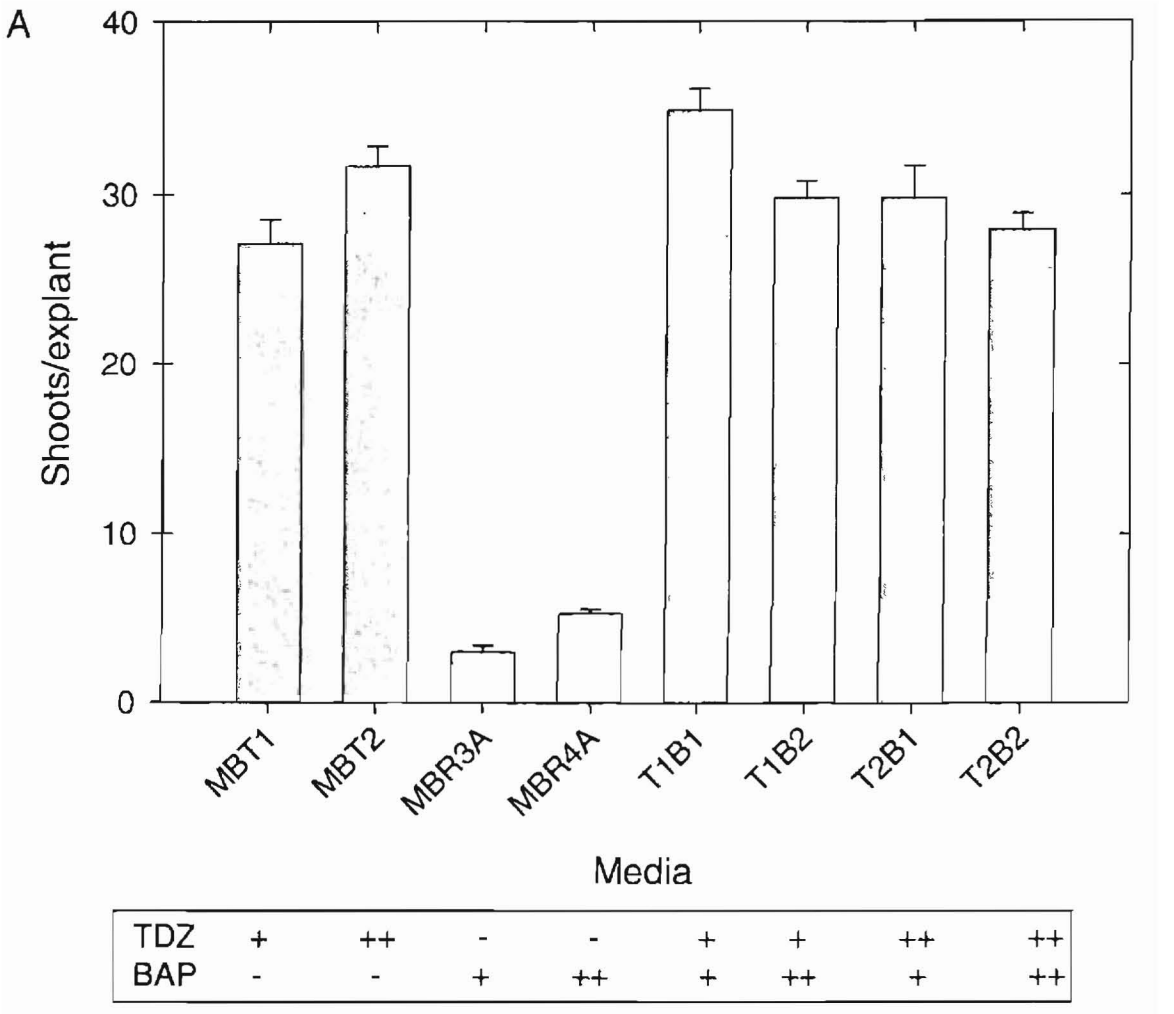


Figure 5.5 Shoot formation in durum wheat (*T. turgidum* AC Plenty). Number of shoots per explant (**A**). Values represent means ± SE, $n = 15$. Symbol (+) represent s1 mg/L of TDZ or BAP, (++) represents 2 mg/L of TDZ or BAP, (-) represents TDZ or BAP was not added to the medium. A representative photograph for explants cultured on media T1B2 (**B**) and MBR3A (**C**) is shown.

5.3.4 Rooting responses of regenerated shoots

Forty-two regenerants of each cultivar (CDC Osprey, CDC Clair, CDC Ptarmigan, AC Plenty and AC Nanda) grown on the 8 different culture media were transferred to the rooting media to assess the response of the various regenerants. Results indicated that only the winter wheat varieties CDC Clair, CDC Osprey and CDC Ptarmigan were able to produce roots, mature and set seed (Table 5.2). Durum wheat cultivar AC Plenty and spring wheat cultivar AC Nanda were unresponsive in the rooting media.

The number of heads, the total number of seeds and the seed weight were also recorded. None of the winter wheat varieties responded well in the rooting media when grown on culture media T2B1. Winter wheat cultivars CDC Clair and CDC Osprey also did not respond well when they were grown on the culture media T1B2. Winter wheat cultivar CDC Osprey produced the maximum number of heads per plant when it was cultured on the media MBR4A and subsequently transferred to the rooting media and transplanted to the pot (Table 5.2). The winter wheat cultivar CDC Ptarmigan produced the highest number of heads per plant when grown on the media MBR3A (Table 5.2). In contrast, CDC Ptarmigan did not respond well in the rooting media when cultured on the growth media MBT2, MBR4A, T1B1 or T2B2 (Table 5.2).

Table 5.2 Quantitative data for regenerants of winter wheat cultivars. Shoots from all cultivars (20 plants of each cultivar) cultured on all shooting media were transferred to rooting media and only those genotypes/cultivars exhibiting rooting were transferred to soil and allowed to set seed.

Media	*Cultivar*	*Total No. of heads*	*Total No. of seeds*	*Seed weight (g)*
MBT1	Clair	5	201	6.15
	Clair	7	250	8.35
	Osprey	7	276	9.17
	Osprey	7	238	7.68
	Ptarmigan	4	193	7.27
MBT2	Clair	6	250	7.55
	Clair	4	99	2.75
	Osprey	8	283	9.15
MBR3A	Clair	5	170	4.01
	Clair	6	213	5.82
	Clair	6	199	7.03
	Clair	7	226	8.84
	Ptarmigan	10	321	7.72
	Ptarmigan	10	380	11.06
	Ptarmigan	7	284	8.78
	Ptarmigan	5	173	5.13
	Osprey	9	277	8.66
	Osprey	5	199	4.52
	Osprey	7	282	7.8
	Osprey	5	218	6.67
MBR4A	Osprey	10	180	3.64
	Osprey	5	204	6.44
	Clair	6	245	7.57
	Clair	3	166	5.18
	Clair	5	244	7.16
	Clair	8	228	8.03
	Clair	6	274	8.31
T1B1	Osprey	6	311	7.9
	Osprey	7	265	8.46
	Osprey	7	229	6.85
	Osprey	6	143	3.71
	Clair	8	175	5.24
T1B2	Ptarmigan	8	342	11.39
	Ptarmigan	5	262	8.98
	Ptarmigan	2	59	1.72
	Ptarmigan	4	140	4.18
T2B2	Clair	5	87	1.74
	Clair	8	208	6.4
	Clair	7	265	6.7
	Clair	8	196	6.89
	Osprey	5	174	5.69
	Osprey	7	248	7.83

5.4 Discussion

The primary goal of this study was to optimize a mature embryo regeneration system for various wheat genotypes. Five varieties of wheat CDC Clair, CDC Osprey, CDC Ptarmigan (all winter wheats), AC Plenty (spring wheat) and AC Nanda (durum wheat) were utilized to assess the response of the mature embryo regeneration of these varieties on different culture media. Two types of cytokinins (TDZ and BAP) were included in the culture media. Multiple shoots were generated from the mature embryos of all the varieties used without an intervening callus phase when the culture media contained TDZ alone or in combination with BAP. However, based on statistical analyses, different cytokinins have shown to have significant variations among different varieties of mature wheat embryos tested with regards to the induction of multiple shoots.

Winter wheat varieties used in this study responded well when grown on culture media containing both TDZ and BAP in various concentrations. The multiple shoot formation in winter wheat mature embryos declined when the culture media was not supplemented with TDZ or if only BAP was used as the cytokinin source. TDZ and BAP have also been used successfully as plant cytokinins for inducing somatic embrogenesis in cereals such as rice (Gairi and Rashid, 2004). In barley, TDZ has also been used to develop multiple shoots from mature embryos (Ganeshan *et al.*, 2003). Studies on plant regeneration from mature and immature embryos of winter wheat varieties using plant growth regulators such as 2,4-D instead of TDZ have shown that 2,4-D induces callus as opposed to multiple shoots (Özgen *et al.*, 1998). The number of calli induced

from mature embryos was also significantly higher than that from immature embryos indicating mature embryos may be a better source of explant (Özgen *et al.*, 1998).

Mature embryos of spring wheat cultivar AC Nanda, used in this study responded well on culture media comprising a combination of TDZ and BAP as plant growth regulators. Li *et al.* (2003) were able to use mature and immature embryos of spring wheat as explants in growth culture media containing TDZ to induce callus, but not multiple shoots. TDZ has successfully been used on barley and wheat immature embryos to promote shoot generation from callus (Shan *et al.*, 2000).

Mature embryos of durum wheat AC Plenty responded well on a culture medium with a combination of TDZ and BAP in this study producing an average of 33 shoots (Fig. 5.1). When scutellum was used as the source of explant in durum wheat, an average of 16 plantlets was produced when the cytokinin BAP was used in the culture media (Bommineni and Jauhar, 1996). The use of scutellum from the durum wheat cultivar AC Plenty in another study produced callus and subsequently produced up to seven shoots per explant (Macs *et al.*, 1996). Recently, the auxin 2,4-D was substituted with Picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) and used to induce callus from scutellum and inflorescences of durum wheat which helped to generate an average of 34 plants per scutellum and 16 plants when inflorescence was used as the explant (He and Lazzeri, 2001). Immature inflorescence of durum wheat has also been used to regenerate an average of 16 shoots per 10 explants (Caswell *et al.*, 2000).

Different plant growth regulators such as Picloram, 2,4-D or Dicamba (3,6-dichloro-*o*-anisic acid) have been used in the growth media to study the callus induction and regeneration capacity from durum wheat cultivars when scutellum was used as explant (Satyavathi *et al.*, 2004). This study demonstrated that there is a cultivar X media interaction and the plant growth regulator Dicamba at 2 mg/L was the best medium for callus induction in the durum wheat cultivar Maier (Satyavathi *et al.*, 2004).

Comparison of the results obtained in this chapter using mature embryos cannot be done directly with other studies using immature embryos, scutellum or inflorescence as explants due to a variety of factors including: media X cultivar interaction, an intervening callus phase that is present in most studies, different stages at which the explants were isolated, and different types of cytokinins used. When TDZ was used as a cytokinin source, it was observed that in cultivars such as AC Nanda (spring wheat) and AC Plenty (durum wheat), the ability for root formation from shoots was suppressed. This is in agreement with the results obtained in this chapter and those by Li *et al.* (2003) where the formation of roots was also suppressed in the presence of high TDZ concentrations. However, Li *et al.* (2003) used calli derived from mature embryos for root formation while this study used multiple shoots directly regenerated from mature embryos. This chapter has shown that mature embryos can be used as an alternative source of explant for regeneration. However, due to the substantial cultivar X media interaction, new cultivars that

are to be used for regeneration need to be evaluated using multiple media with different concentrations of cytokinins to obtain optimum results for regeneration.

6.0 SUMMARY AND CONCLUSIONS

LT-tolerance is a complex quantitative trait governed by complex interactions of many genes. Genetic studies on LT tolerance in cereals such as barley, wheat and rye have identified group 5 chromosomes as the major contributor towards alleviation of LT-stress. Chromosome 5A in wheat is also involved in the regulation of *wcs120* and *wcor410* gene families involved in LT tolerance. The vernalization locus in wheat, *vrn-1* is also present on chromosome 5A. However, genetic studies have indicated at least 15 chromosomes may be involved in contributing towards the alleviation of LT stress (Sutka, 1994). Hence, the primary objective of this study was to identify chromosomal regions in wheat contributing towards LT-tolerance.

In this study, a doubled haploid (DH) population was developed from the two winter wheat parents Norstar and Cappelle-Desprez. Norstar and Cappele-Desprez were chosen as parents for developing the mapping population since they have the same vrn-1 region on chromosome 5A and may help in differentiating LT-tolerance from vernalization. In an effort to identify the genomic regions involved in LT-tolerance, Microsatellite and AFLP markers were used to construct a genetic map of Norstar X Cappelle-Desprez which spanned 2292 cM. A major QTL on chromosome 5A for LT-tolerance contributing 80% of the LT-tolerance trait was mapped in the Norstar X Cappelle-Desprez population indicating that chromosome 5A plays a key role in LT-tolerance. Single marker analysis also identified 1B, 1D, 4A, 5B and 5D chromosomes as possibly playing

a minor role towards LT-tolerance in the population derived from the Norstar X Cappelle-Desprez cross.

The results obtained in this study are in agreement with results obtained by other researchers in cereals. In *Triticum monococcum*, *Fr-A1* and *Fr-A2* are the major frost tolerance loci that have been mapped to chromosome 5A (Vagujfalvi *et al.*, 2000; 2003). In barley, a major QTL (*Fr-H1*) for LT-tolerance has been mapped to the 5H chromosome which is responsible for 62% of the variation with regards to LT tolerance (Skinner *et al.*, 2005). An additional frost tolerance locus (*Fr-H2*) has been reported in barley (Francia *et al.*, 2004; Skinner *et al.*, 2006). The QTL identified in the Norstar X Cappelle-Desprez cross corresponds to the *Fr-A2* locus in diploid and hexaploid wheat (Vagujfalvi *et al.*, 2000; 2003). The mapping results obtained using different mapping populations in cereals such as wheat, barley and rye have not only implicated the group 5 chromosomes as prime candidates for genes responsible for alleviating LT-stress, but also suggest a possible conservation of these genes in the course of evolution of cereals.

Recently, the discovery of C-repeat binding factors (CBF) as the key transcription factors that induce cold-regulated genes has paved the way to understand the regulation of cold-regulated genes. The presence of CBF transcription factors in both monocotyledonous and dicotyledonous plants suggests that CBFs are conserved. Studies aimed at mapping the *CBF* genes in monocots and dicots have revealed that the *CBF* genes are present in clusters. In Arabidopsis, the three *CBF* genes mapped are present in a tandem cluster on

chromosome 4 (Gilmour *et al.*, 1998). Mapping experiments to elucidate the location of CBF genes in cereals have yielded interesting results. In cereals such as barley, wheat and rye orthologs of Arabidopsis *CBF* genes have been mapped. In barley, the gene encoding for a transcription factor HvCBF3 that is induced by low-temperature has been mapped to chromosome 5H (Choi *et al.*, 2002). Recently, 20 *CBF* genes have been mapped on the long-arm of chromosome 5H in barley (Skinner *et al.*, 2006). Comparative mapping with Triticeae LT-tolerance QTLs have indicated that some of the *CBFs* identified in barley may be suitable candidates for the *Fr-H2* QTL identified (Skinner *et al.*, 2006). Similarly, in diploid wheat *Triticum monococcum*, 11 CBF genes have been identified and mapped to the *Fr-A^m2* region on chromosome 5A (Miller *et al.*, 2006). In addition, *TmCBF18* has been mapped to chromosome 6A and *TmCBF5* has been mapped to chromosome 7A in *Triticum monococcum* (Miller *et al.*, 2006). In hexaploid wheat, studies using frost-tolerant and frost-sensitive wheat varieties have implicated chromosome 5A as the possible location of potential *CBF* genes that map to the *Fr-A2* locus conferring increased low-temperature tolerance (Vagujfalvi *et al.*, 2005). Identification of a major QTL for low-temperature tolerance in the Norstar X Cappelle-Desprez cross similar to the *Fr-A2* locus in diploid wheat suggested that a cluster of *CBF* genes could be present at this locus as identified by other studies.

In this study, the Norstar BAC library was screened to identify the number of potential CBF genes. *Hin*dIII restriction profiles obtained from the isolated CBF clones indicate that the hexaploid wheat Norstar may have at least 52 or

more *CBF* genes. Preliminary studies have provided an insight of the CBF clones that may map to the *Fr-A2* locus, however, future studies may help in identifying the clusters of *CBF* genes that map to the *Fr-A2* locus and other potential *CBF* genes that may not be present at the *Fr-A2* locus. Studies in barley have indicated that the regulators of CBF genes, *ICE1* and *FRY1* have been mapped to the chromosome 7H where no QTL for LT-tolerance have been identified suggesting that the *CBF* genes may be a better candidate gene for LT-tolerance. It is possible that the allelic differences in the *CBF* genes may be the major contributing factor for the difference observed in the LT-tolerance potential (Tondelli *et al.*, 2006). It has to be noted that while Norstar is one of the most cold-hardy hexaploid wheats, other cereals such as rye have more LT-tolerance potential than Norstar or any of the wheat or barley relatives. Studies aimed at understanding the differences in low temperature tolerance potential in rye may offer clues as to how rye has better LT-tolerance potential. It may be possible to identify and transfer the genes responsible for LT-tolerance in order to break the current threshold of LT-tolerance potential limitation present in wheat and barley.

The mapping studies undertaken in this thesis and other studies have provided insight into the chromosomal regions involved in LT-tolerance in cereals and implicated the group 5 chromosomes. Future studies should concentrate on identifying the specific genes involved in the 5A region of wheat which contribute to LT-tolerance. The results obtained by this study and other research groups point out that the *CBF* genes located on the 5A chromosome may hold the key for better understanding of the complexity of gene interactions involved during LT

exposure. Hence, it will be worthwhile to identify differences in this region using molecular markers in the existing germplasm of cereals. This may lead to identification of genetic stocks in the existing germplasm that may be used for breeding superior LT-tolerant crops. However, it should be noted that in barley, the regulators of *CBF* genes are not located on the group 5 chromosomes and care should also be taken to identify and map the regulators of *CBF*s. It may also be possible that certain less-hardy wheat cultivars may have better candidate genes contributing to LT-tolerance, but may lack the regulators for the expression of these genes. Therefore, a wide range of both hardy and less-hardy germplasm of wheat should be screened in order to identify all the potential genes and their regulators to understand and identify the best combination that might alleviate LT stress. Future studies in the more cold-hardy cereals, such as rye, will also help in identifying various regulators and genes involved in LT-tolerance which could be applied to other cereal crops.

The CBF clones identified in this study need to be sequenced and assembled to understand how these genes are organized on the chromosome. It will also allow identification of the allelic differences present in the *CBF* genes and also provide an estimation of the number of *CBF* genes present on chromosome 5A (and on other chromosomes). Gene-specific primers can be designed using the identified *CBF* gene sequences and used to screen the existing germplasm of wheat to identify other alleles present. The primers can also be used in the Norstar X Capelle-Desprez mapping population developed to identify and map the chromosomal locations of all the potential *CBF* genes. It is

important to identify differences in the existing germplasm for LT-tolerance for breeding new LT-tolerant cultivars due to the tremendous opposition towards genetically modified crops.

Unlike Arabidopsis, cereals such as wheat and barley are not always amenable to transformation and tissue culture. A suitable transformation and tissue culture system for both winter and spring wheats is required for studies using genes implicated in LT-tolerance. The work in this study has demonstrated that mature embryos of wheat can be successfully used for regeneration. In addition, growth media containing different concentrations and types of cytokinins were tested to identify the best media requirement for specific cultivars. Results obtained in this study show the importance of media X cultivar interaction, as spring and durum wheat cultivars did not respond as well as the winter wheat cultivars for rooting. For the purpose of transformation, instead of using the media with the ability to produce more shoots, a suitable regeneration medium should be used which does not hinder the ability of the plants to root. When regenerants were grown on media lacking TDZ, most plants had better root formation which has also been confirmed by other studies (Li *et al.*, 2003). This is an important aspect to consider as the transformation efficiency in cereals is very low, and therefore, it would be better to use a medium which does not interfere with the rooting process as opposed to concentrating on maximizing the number of shoots produced per explant. It is envisioned that the present study will serve as a platform for helping to better understand the genetics underlying

LT-tolerance in wheat and help in developing better cultivars with increased LT-tolerance potential, thereby assisting in the expansion of winter-wheat cultivation.

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Appendix A Composition of solutions

2x loading buffer
95% (v/v) formamide
10 mM NaOH
0.05% (w/v) bromophenol blue
0.05% (w/v) xylene-cyanol blue

Church buffer (1X SSC)
0.15 M NaCl
0.15 M Na-citrate, pH 7.0
0.1% (w/v) sodium dodecyl sulphate (SDS)

Denaturation solution
0.5 M NaOH
1.5 M NaCL

Developer solution
2.5 mL 37% (v/v) formaldehyde
60 g sodium carbonate in 2 L of H_2O

*EcoR*I buffer (1X)
50 mM NaCl
100 mM Tris-HCl
10 mM $MgCl_2$
0.025% Triton X-100, pH 7.5

Extraction buffer
50 mM Tris-HCl, pH 8.0
0.7 M NaCl
10 mM EDTA
1% (w/v) cetyltrimethylammonium bromide (CTAB)

Fix/stop solution
10% (v/v) acetic acid

Gel binding solution
3 µL bind silane in 1 mL 95% (v/v) ethanol
0.5% (v/v) glacial acetic acid

LB plate
10g tryptone
5g, yeast extract
5g NaCL
15g agar in 1L of H_2O, pH 7.5

LB broth
10g tryptone
5g yeast extract
5g NaCL in 1L of H_2O pH 7.5

Loading dye (6X)

10mM Tris-HCl (pH 7.6)
0.03% bromophenol blue
60mM EDTA
60% glycerol

Neutalization solution
1.5 M NaCL,
0.5 Tris-HCL, pH 8.0

PCR reaction buffer
10 mM Tris-HCl, pH 8.3,
50 mM KCl
1.1mM $MgCl_2$
0.01% (w/v) gelatine

Precipitation buffer
50 mM Tris-HCl, pH 8.0
10 mM EDTA
1% (w/v) CTAB

SSPE
0.75 M NaCl
50 mM NaH_2PO_4
5 mM EDTA, pH 7.4

Staining solution
2.5 mL 37% (v/v) formaldehyde
2 g silver nitrate in 2 L of H_2O

TAE buffer (10 X)
0.4 M Tris-acetate,pH 8.0
0.01 M EDTA
0.2 M Acetic acid

TBE buffer (10 X)
0.89 M Tris
0.02 M EDTA
0.89 M Boric acid

TE buffer
10 mM Tris-HCl, pH 8.0
1 mM EDTA

Appendix B Comparision of SSR markers mapped to other published maps

Primer	Norstar X Cappelle	Synthetic X Opata	Consensus	Composite
gwm2_235	3A	3A, 3D	3A, 3D	3A
gwm6_(201C)	4B	4B	4B	4B, 4A
gwm6_200	5A			
gwm6_202	4B			
gwm11_(200C)	1B	1B	1B	1B
wmc28_191	5B		5B	5B
gwm43_182	7B	7B	7B	
gwm44_181	7D	7D	7D	7D
gwm46_186	7B	7B	7B	7B
gwm47_(160C)	2B	2A, 2B	2A, 2B	2B
gwm55_111	1D	2B, 6D	2B,6D	2B,6D
gwm55_127	2B	2B,6D	2B,6D	2B,6D
gwm55_128	unlinked			
gwm55_170	2B			
gwm58_135	6B			
gwm63_(212C)	2A	7A	7A	
gwm63_177	2D			
gwm63_197	2A			
gwm71_(136C)	2A	2A, 3D	2A, 3D	2A
gwm71_115	2D			
gwm71_128	2A			
gwm102_161	2D	2D	2D	
gwm106_(134C)	1D	1D	1D	
gwm108_128	3B	3B	3B	3B
gwm122_147	2A	2A	2A	2A
gwm129_130	5A	2B, 5A	2B, 5A	
gwm130_130	7D	7A	7A	7A
gwm131_123	1B	1B, 3B	1B, 3B	
gwm133_(122C)	3A	6B	1B,3A,4D,5B,6B,6D	6B
gwm133_(138C)	6B			
gwm133_(190C)	7B			
gwm133_114	3A			
gwm133_70	4D			
gwm135_127	1A	1A	1A	1A
gwm140_197	1B	1B	1B	1B
gwm146_181	7A	7B	7B	7B
gwm148_148	2B	2B	2B	
gwm149_138	4B	4B	4B	
gwm153_(176CM)	1B			1B
gwm154_100	5A	5A	5A	5A
gwm156_289	5A	5A	5A	

gwm161_153	3D	3D	3D	
gwm165_194	4D	4A,4B,4D	4A,4B,4D	
gwm165_197	4A			
gwm165_263	4B			
gwm169_(195CM)	6A	6A	6A	
gwm174_179	5D	5D	5D	
gwm181_126	3B	3B	3B	
gwm186_122	5A	5A	5A	
gwm190_211	5D	5D	5D	
gwm191_(168C)	6B	2B,5B,6B	2B,5B,6B	
gwm192_137	4B	5D	5D	4D
gwm192_142	4D			
gwm194_138	4D	4D	4D	
gwm200_168	6D			
gwm205_158	5A	5A,5D	5A,5D	
gwm212_102	5D	5D	5D	
gwm213_158	5B	5B	5B	
gwm218_148	3A			3A
gwm234_238	5B	5B	5B	5B
gwm247_155	3B	3B	3B	
gwm257_197	2B	2B	2B	
gwm259_105	1B	1B	1B	
gwm261_208	2D	2D	2D	
gwm264_171	1B	1B	1B, 3B	1D
gwm264_218	3B	1B	1B	1D
gwm265_(199CM)	3D	2A	2A	
gwm268_254	1B	1B	1B	
gwm269_(200C)	5D	5D	5D	5D
gwm272_138	5D	5D	5D	
gwm273_169	1B	1B	1B	1B
gwm276_183	unlinked	7A	7A	7A
gwm282_259	7A	7A	7A	
gwm292_209	5D	5D	5D	
gwm293_175	5A	5A	5A	
gwm294_76	2A	2A	2A	
gwm295_248	7D	7D	7D	
gwm296_(176C)	2A	2A, 2D	2A,2D	7D
gwm296_150	2D	2A,2D	2A,2D	7D
gwm296_177	2A			
gwm297_153	7B	7B	7B	
gwm299_(190CM)	3B	3B	3B	3B
gwm304_214	5A	5A	5A	2A,5A
gwm312_192	2A	2A	2A	
gwm325_142	6D	6D	6D	6D
gwm332_(206C)	7A	7A	7A	7A

gwm334_334	6A	6A	6A	
gwm339_166	2A	2A	2A	
gwm340_125	3B	3B	3B	
gwm341_208	3D	3D	3D	3D
gwm344_128	7A	7B	7B	
gwm349_(215CM)	2D	2D	2D	2D
gwm349_226	2D			
gwm350_136	7A	7A	7A	4A
gwm356_208	3D	2A	2A	
gwm358_165	5D	5D	5D	
gwm359_209	2A	2A	2A	2A
gwm361_137	6B	6B	6B	
gwm368_234	4B	4B	4B	4B
gwm369_187	3A	3A	3A	
gwm371_193	5B	5B	5B	
gwm375_224	4B			
gwm383_187	3D	3D	3D	3D
gwm389_130	3B	3B	3B	
gwm397_188	4A	4A	4A	
gwm408_184	5B	5B	5B	
gwm410_241	4D	2B	2B	
gwm410_271	5A			
gwm410_345	2B			
gwm425_146	2A	2A	2A	
gwm427_202	6A	6A	6A	
gwm429_201	2B	2B	2B	
gwm437_114	7D	7D	7D	
gwm443_138	5A	5B	5B	
gwm448_222	2A	2A	2A	2A
gwm448_246	2D			
gwm448_251	4B			
gwm456_133	3D	3D	3D	
gwm458_111	1D	1D		
gwm459_188	6A	6A	6A	
gwm469_169	6D	6D	6D	
gwm484_150	2D	2D	2D	
gwm498_137	1A	1A, 1B		1B
gwm498_161	1B			
gwm499_152	5B	5B	5B	
gwm501_178	2B	2B	2B	
gwm513_146	4B	4B	4B	
gwm538_(150CM)	4B	4B	4B	
gwm547_178	3B	3B	3B	
gwm547_190	3B			
gwm565_(200C)	5D	5D	5D	

gwm570_134	6A	6A	6A	
gwm570_140	6A			
gwm577_150	7B	7B	7B	
gwm583_165	5D	5D	5D	
gwm604_143	5B	5B	5B	
gwm608_(136C)	6B	2D, 4D	2D,4D,1B,1D,6B	
gwm608_(142C)	4D			
gwm608_143	4D			
gwm608_158	2D			
gwm611_176	7B	7B	1A,1B	
gwm614_(122C)	2A			
gwm614_150	2A	2A	2A	2A
gwm614_153	2D			
gwm617_175	5A	5A,6A	5A,6A	
gwm635_107	7A	7D		
gwm636_103	2A	2A		
gwm639_(138C)	5A	5A	5A	5A
gwm639_166	5B			
gwm644_134	6B	6B,7B	6B,7B	6B
gwm644_151	7B			
gwm645_162	3D	3D	3D	
gwm666_119	5A	1A,3A,5A,7A	1A,3A,5A,7A	3A
gwm674_166	3A	3A	3A	
wmc11_(163C)	3A		1A,3A,3D	3D
wmc11_183	3A			
wmc11_243	3D			
wmc14_243	7D		7D	
wmc24_140	1A		1A	1A
wmc41_166	2D		2D	
wmc44_263	1B		1B	
wmc48_141	4D		4A,4B,4D	
wmc48_189	4B			
wmc52_219	4D		4D	
wmc59_197	1A			
wmc69_(225C)	2D			
wmc69_263	1B			
wmc74_236	5A			
wmc74_254	4D		4D	
wmc74_260	unlinked			
wmc75_205	5B		5B	
wmc85_(227C)	1B			1B
wmc85_220	3B			
wmc89_136	4D		4A,4B,4D	
wmc89_185	4B			

wmc93_172	1D		1A,1D	
wmc93_176	1A			
wmc94_111	7D		7D	
wmc95_(161C)	1A		1A,5D	
wmc104_140	6B		6B	1A
wmc105_352	6B		6B	
wmc107_179	3A			
wmc111_238	2D		2D	
wmc113_(334C)	6D		1B	6D
wmc113_352	7B			
wmc113_411	6A			
wmc113_437	1B			
wmc116_394	7A			
wmc118_174	5B		5B	5B
wmc145_295	3B			
wmc149_159	5B		2A,2B,5B	
wmc149_205	2B			
wmc153_181	3A		3A	1D,3A
wmc154_152	2B		2B	2B
wmc157_104	7B		7D	7D
wmc167_188	2D		2D	
wmc168_322	7A		7A	
wmc175_252	2B		2B,2D	
wmc177_189	2A		2A	
wmc182_177	6B		3B,4D,6A,6B,7A,7B, 7D	
wmc201_248	6A		6A	6A
wmc206_(156C)	3A		1B,3B,4D,5D,6A	4B,5D
wmc206_(164C)	1B			
wmc206_(184C)	unlinked			
wmc206_(197CM)	2A			
wmc206_(212CM)	5A			
wmc206_171	5B			
wmc206_243	4B			
wmc219_161	4A		4A	
wmc222_151	1D		1D	
wmc222_166	unlinked			
wmc233_262	5D		5D	
wmc243_(178C)	2D		2B,2D,6A	
wmc243_180	2D			
wmc245_142	2B		2B,2D	
wmc247_212	2A			5B
wmc247_235	7A			
wmc247_280	2A			
wmc247_496	5B			

wmc247_82	7A			
wmc256_117	6A		6A	
wmc257_334	2B		2B	2B
wmc261_147	2A		1D,2A,2B,3B,7B	
wmc262_205	4A		4A	
wmc269_(75CM)	2B			
wmc269_106	3A			
wmc273_240	7A	7A(gupta)	7B,7D	
wmc276_337	7B		7B	
wmc283_117	7A		4A,7A	
wmc283_99	4A			
wmc285_(306C)	4D		4D	
wmc285_298	4D			
wmc326_211	3B		3B,5B	3B
wmc327_183	5A	5A	5A	
wmc331_132	4D		4D	
wmc357_204	5D		5D	5D
wmc382_(169C)	2B		2A,2B	
wmc388_(163C)	3A		3A,5A,7A	3A,6A
wmc388_174	5A			
wmc398_180	6B		6A,6B	
wmc405_216	7A		1D,5B,5D,7A,7D	7D
wmc415_177	5B		5A,5B	
wmc418_262	3B			
wmc419_160	1B		1B,4B,6B	4D
wmc429_231	1D		1D	1D
wmc445_227	2B		5A	2B,2D
wmc445_244	2D			
wmc445_257	3D			
wmc453_184	2A			
wmc457_163	4D		4D	4D
wmc463_152	7D		7D	
wmc473_145	4D		4D,6B,7D	4D,7D
wmc487_(156C)	6B		6B	
wmc487_171	6B			
wmc488_139	7D		7A,7D	
wmc489_(275C)	5A		1D,2B,3A,4D,5A,7D	
wmc492_151	3D		3D,5A	
wmc494_212	6B		6B	
wmc497_222	4A		4A,7A	
wmc505_118	3A		3A,3B,3D	
wmc505_138	3B			
wmc517_(203C)	7B		7B	
wmc517_192	7B			
wmc522_(174CM)	2A		2A	

wmc524_212	5A		5A	
wmc537_183	5B		5B	
wmc590_215	1D		1D	
wmc597_227	7A		1B,2B,3B,4A,6B	
wmc597_241	2B			
wmc617_213	4B		4A,4B,4D	
wmc617_240	4A			
wmc627_(202C)	6A		2B,3A	
wmc627_457	5B			
wmc671_125	6A		7D	
wmc710_194	7A			
wmc716_121	3B		1A	
wmc716_149	1A			
wmc719_240	4B		1B	
wmc720_122	4D		4D	
wmc773_(111CM)	6D		5B,6D	
barc3_194	6A	6A	6A	6A
barc4_194	5B	5B	5B	
barc20_(192C)	4B	7B	4B	7B
barc20_178	4B			
barc23_333	5B			
barc40_218	5A	5A	5A	5A
barc52_126	4A	4A	3D	4A
barc56_117	5A		5A	
barc59_(182C)	5B		2D,5B	5B
barc59_171	5B			
barc74_(183C)	5B	5B		5B
barc74_171	5B			
barc76_209	7D		2A,6B,7D	7D
barc77_(172C)	3B	3B	3B	
barc77_234	3B	3B	3B	
barc81_200	1B	1B	1B	1B
barc87_(102CM)	3B		3B,7D	
barc89_(137C)	5B	5B	5B	5B
barc89_134	5B		5B	5B
barc91_140	2B	2B	4D	2B
barc94_(245C)	2B		7B	
barc94_234	2B			
barc97_244	7D			
barc100_167	5A	5A		5A
barc105_(143C)	4D	7D		7D
barc105_154	4D			
barc108_161	7A	7A	7A	
barc110_194	5D			5D
barc114_131	4D			

barc115_196	3B			
barc119_225	1A	1A,1D	1A,1D	1A,1D
barc122_236	5A	5A	2D	
barc126_127	7D	7D	7D	7D
barc127_(203C)	7A	7A	6B,7A	
barc127_193	7A			
barc133_117	5B	3B		3B
barc134_193	6B	6B	6B	6B
barc135_(251C)	7D	3D		5A
barc135_244	7D			
barc140_144	5B	5B	5B,5D	5B
barc141_249	5A	5A	5A,6B	5A
barc141_260	5A			
barc143_233	5D	5D	5D	5D
barc144_(227C)	5D	5D	5D	5D
barc144_246	5D			
barc146_134	6B			
barc149_135	1D	1D	1D	
barc153_(232C)	7D	4A		4A
barc153_226	7D			
barc154_230	7A	7A	7A,7D	7D
barc169_(124C)	1D	1D	1D	1D
barc169_122	1D			
barc171_237	6A			
barc172_176	7D	7D	7D	7D
barc175_225	6D	6D	6D	6D
barc177_156	5D	5D		5D
barc180_199	5A	5A	3B,5A	5A
barc183_(168CM)	6D	6D	2B,6D	6D
barc186_200	5A	5A	5A	5A
barc186_210	5A			
barc188_214	1B	1B	1B	1B
barc195_229	7A	6A	6A,7A	6A
barc197_193	5A			
barc206_224	4A	4A	3B,4A,6A	4A
barc216_99	5B	5B		5B
barc217_108	4D	4D		4D
barc218_(226C)	3B	3B		3B
barc218_(232C)	3A			
barc218_242	3B			
barc228_612	2D	2D	2D	2D
barc229_(187C)	1D	1D	1D,3B	1D
barc229_(250C)	3B			
barc229_209	3B			
barc230_206	2B	5A		5A

barc231_251	7A	2A		2A
barc232_218	5B		5A,5B,5D	5B
barc263_210	1A	1A		1A
barc271_163	1D	1D		1D
barc275_266	5B	7A		
barc292_199	7A	2D,4B,7A		2D,4B
barc303_218	5A	5A		5A
barc309_154	2A	2A		2A
barc322_237	5D	5D		5D
barc334_(175C)	4D	4D		4D
barc334_171	4D			
barc340_212	4B	5B,7B		5B,7B
barc346_200	2D	1D		1D
barc349_(99C)	2D	2B		2B
barc349_97	5B			
barc353_222	6A	2A		2A
barc353_231	2A			
barc356_148	3A	3A		3A
barc360_304	5A	5A		5A
barc361_281	6B	2B,5D		2B,5D
barc1121_125	6D	6D		6D
barc1124_(118C)	3B			
cfd2_306	2A			
cfd2_326	5A			
cfd2_366	5B			
cfd4_221	3B		3B,3D	3B
cfd5_183	6D		5B	6D
cfd7_213	5B		5B,5D	5D
cfd7_236	5D			
cfd8_158	5D		5D	5D
cfd9_199	3B			
cfd18_174	5D			
cfd19_272	1D		1D,5D,6D	
cfd19_316	6D			
cfd20_308	1B		5B,7A	1B
cfd21_259	1D			
cfd30_219	3A			
cfd31_216	7D		7D	7D
cfd33_168	6D			6D
cfd37_192	5D		5D,6D	6D
cfd37_211	6D			
cfd43_176	2D		2D	2D
cfd48_227	1B		1B,1D	
cfd55_265	3D			3D
cfd59_277	1A		1A,1B,1D	1B,1D

cfd62_222	3D		2D,3D	2D
cfd70_122	3D		2B,3D,3D	3D
cfd76_153	6D		6D	6D
cfd80_164	6A		6A,6D	6D
cfd116_228	2D		2D	2D
cfd152_292	3D		3D	3D
cfd152_304	3A			
cfd168_217	2D			
cfd188_257	6D		6D	6D
cfa2028_257	7A		7A	7A,7B
cfa2104_310	5A		5A,5D	5D
cfa2123_243	7A			
cfa2129_154	1A			
cfa2141_248	5A			
cfa2147_332	1B		1B,1D	1B,1D
cfa2155_217	5A		5A	
cfa2163_162	5A		5A	
cfa2170_204	3A			
cfa2193_207	3A		3A	3A
gdm33_(136CM)	1D	1D	1A,1B,1D	
gdm33_127	1A			
gdm33_170	1D			
gdm67_131	7D	7D	7D	7D
gdm88_122	7D		7D	
gdm109_(168C)	5A		5A	
gdm109_(172C)	3D			
gdm109_180	5A			
gdm109_191	3D			
gdm150_119	7D	7D		
gpw93003_233	7B			
gpw93013_190	1B			

Appendix C Unique SSR markers.

Marker	*Chromosome*	*size (bp)*
gwm6_200	5A	200
gwm55_111	1D	111
gwm55_128	unlinked	128
gwm63_(212C)	2A	212
gwm63_177	2D	177
gwm63_197	2A	197
gwm71_115	2D	115
gwm130_130	7D	130
gwm146_181	7A	181
gwm192_137	4B	137
gwm264_218	3B	218
gwm265_(199CM)	3D	199
gwm344_128	7A	128
gwm356_208	3D	208
gwm369_187	3A	187
gwm410_241	4D	241
gwm410_271	5A	271
gwm443_138	5A	138
gwm448_246	2D	246
gwm448_251	4B	251
wmc74_236	5A	236
wmc85_220	3B	220
wmc113_352	7B	352
wmc113_411	6A	411
wmc116_394	7A	394
wmc145_295	3B	295
wmc157_104	7B	104
wmc206_(156C)	3A	156
wmc206_(197CM)	2A	197
wmc206_(212CM)	5A	212
wmc206_171	5B	171
wmc222_166	unlinked	166
wmc247_212	2A	212
wmc247_235	7A	235
wmc247_280	2A	280
wmc247_82	7A	82
wmc269_(75CM)	2B	75
wmc445_257	3D	257
wmc597_227	7A	227
wmc627_(202C)	6A	202
wmc627_457	5B	457
wmc671_125	6A	125

wmc710_194	7A	194
wmc716_121	3B	121
wmc719_240	4B	240
barc23_333	5B	333
barc94_(245C)	2B	245
barc94_234	2B	234
barc135_(251C)	7D	251
barc135_244	7D	244
barc197_193	5A	193
barc218_(232C)	3A	232
barc275_266	5B	266
barc349_(99C)	2D	99
barc349_97	5B	97
barc353_222	6A	222
cfd2_306	2A	306
cfd9_199	3B	199
cfd30_219	3A	219
cfd152_304	3A	304
cfa2170_204	3A	204
cfa2173_254	unlinked	254
gdm109_(172C)	3D	172
gdm109_191	3D	191

Appendix D Chromosome locations of mapped AFLP markers

AFLP marker	*No.of polymorphic bands*	*Markers mapped*	*chromosome location*	*Allele size (bp)*
E32M47	11	E32M47_370	1A	370
		E32M47_(100C)	1A	100
		E32M47_(121C)	2B	121
		E32M47_166	5B	166
		E32M47_445	6A	445
		E32M47_(144C)	6A	144
		E32M47_146	6A	146
		E32M47_304	6A	304
		E32M47_(92C)	6B	92
		E32M47_(187C)	6B	187
		E32M47_(242C)	7B	242
E32M48	8	E32M48_(182CM)	7B	182
		E32M48_136	7A	136
		E32M48_(119C)	7B	119
		E32M48_65	3B	65
		E32M48_(193CM)	4B	193
		E32M48_75	5B	75
		E32M48_242	5B	242
		E32M48_(110CM)	6B	110
E32M49	5	E32M49_(271C)	6B	271
		E32M49_(55C)	7D	55
		E32M49_159	2B	159
		E32M49_(213C)	2B	213
		E32M49_100	3B	100
E32M50	3	E32M50_(117C)	4A	117
		E32M50_(218C)	unlinked	218
		E32M50_(150C)	unlinked	150
E32M59	3	E32M59_(154CM)	2B	154
		E32M59_238	2B	238
		E32M59_168	4B	168
E36M47	8	E36M47_217	4B	217
		E36M47_(136C)	5B	136
		E36M47_138	7B	138
		E36M47_(360C)	1A	360
		E36M47_428	1B	428
		E36M47_144	2D	144
		E36M47_124	3B	124
		E36M47_107	3B	107
E36M49	9	E36M49_(254CM)	5B	254
		E36M49_(185C)	6B	185

		E36M49_(150)	6D	150
		E36M49_(55C)	7A	55
		E36M49_138	2D	138
		E36M49_274	7A	274
		E36M49_189	7B	189
		E36M49_314	7B	314
		E36M49_57	1B	57
E36M50	3	E36M50_198	4A	198
		E36M50_(190CM)	7B	190
		E36M50_(83C)	1A	83
E36M59	8	E36M59_107	3B	107
		E36M59_(117C)	3B	117
		E36M59_(181C)	3D	181
		E36M59_(265CM)	3D	265
		E36M59_340	4A	340
		E36M59_204	4A	204
		E36M59_104	4B	104
		E36M59_229	7B	229
E36M60	8	E36M60_(84C)	1B	84
		E36M60_(177C)	2B	177
		E36M60_(478C)	3B	478
		E36M60_133	3D	133
		E36M60_(108C)	4B	108
		E36M60_270	5D	270
		E36M60_191	6A	191
		E36M60_(95C)	7A	95
E36M61	11	E36M61_(117CM)	7A	117
		E36M61_191	unlinked	191
		E36M61_93	1B	93
		E36M61_170	2B	170
		E36M61_81	2B	81
		E36M61_(209C)	2D	209
		E36M61_(226C)	3D	226
		E36M61_(159C)	4A	159
		E36M61_318	5A	318
		E36M61_803	5A	803
		E36M61_116	6A	116
E36M62	1	E36M62_(204)	4D	204
E37M47	13	E37M47_84	6A	84
		E37M47_360	6B	360
		E37M47_(158CM)	7A	158
		E37M47_(181C)	7A	181
		E37M47_(161C)	1B	161
		E37M47_277	1B	277
		E37M47_169	1B	169

		E37M47_(263)	2B	263
		E37M47_64	2B	64
		E37M47_(143C)	2D	143
		E37M47_87	3B	87
		E37M47_66	3B	66
		E37M47_(346C)	5A	346
E37M48	10	E37M48_(437C)	5A	437
		E37M48_92	6B	92
		E37M48_(162CM)	7A	162
		E37M48_71	7B	71
		E37M48_158	7B	158
		E37M48_(154CM)	7B	154
		E37M48_(68C)	1A	68
		E37M48_(67C)	1A	67
		E37M48_(180C)	2D	180
		E37M48_295	3A	295
E37M49	8	E37M49_(77C)	2D	77
		E37M49_215	3B	215
		E37M49_(199CM)	3D	199
		E37M49_(232C)	5B	232
		E37M49_(260C)	5D	260
		E37M49_(108CM)	7B	108
		E37M49_(148C)	unlinked	148
		E37M49_159	1A	159
E37M50	2	E37M50_(128C)	1A	128
		E37M50_272	7A	272
E37M59	6	E37M59_(81CM)	7A	81
		E37M59_(191C)	3A	191
		E37M59_175	3B	175
		E37M59_140	4A	140
		E37M59_300	4A	300
		E37M59_196	6A	196
E37M60	5	E37M60_(49C)	6A	49
		E37M60_73	7B	73
		E37M60_(123C)	2D	123
		E37M60_(161CM)	3D	161
		E37M60_193	5B	193
E37M61	4	E37M61_169	7A	169
		E37M61_284	1B	284
		E37M61_99	3B	99
		E37M61_(120C)	3D	120
E37M62	3	E37M62_(72C)	6A	72
		E37M62_197	7B	197
		E37M62_79	2B	79
E40M59	8	E40M59_(228C)	2D	228

		E40M59_67	2D	67
		E40M59_277	3B	277
		E40M59_(257C)	4A	257
		E40M59_260	4A	260
		E40M59_(408C)	7A	408
		E40M59_(130C)	unlinked	130
		E40M59_224	1A	224
E40M60	10	E40M60_(158C)	1A	158
		E40M60_88	1B	88
		E40M60_198	2B	198
		E40M60_(66C)	2D	66
		E40M60_375	3A	375
		E40M60_(673C)	5A	673
		E40M60_87	5B	87
		E40M60_(273CM)	6B	273
		E40M60_80	7A	80
		E40M60_126	unlinked	126
E40M61	6	E40M61_(264C)	1A	264
		E40M61_251	2D	251
		E40M61_284	5B	284
		E40M61_(116CM)	6B	116
		E40M61_50	6D	50
		E40M61_(249C)	unlinked	249
E40M62	5	E40M62_132	3A	132
		E40M62_(359C)	3B	359
		E40M62_136	4A	136
		E40M62_153	5B	153
		E40M62_(273C)	6A	273
E41M48	9	E41M48_(378CM)	1B	378
		E41M48_(151CM)	2B	151
		E41M48_(184C)	3A	184
		E41M48_64	3D	64
		E41M48_106	5A	106
		E41M48_226	5B	226
		E41M48_136	7A	136
		E41M48_147	7A	147
		E41M48_(133C)	7A	133
E41M49	7	E41M49_(179C)	3B	179
		E41M49_456	3D	456
		E41M49_88	5B	88
		E41M49_312	6A	312
		E41M49_105	6B	105
		E41M49_(89C)	7A	89
		E41M49_(90C)	unlinked	90
E41M50	5	E41M50_(105C)	1B	105

		E41M50_47	3B	47
		E41M50_131	5B	131
		E41M50_169	6A	169
		E41M50_318	7A	318
E41M59	12	E41M59_141	1A	141
		E41M59_115	1D	115
		E41M59_46	2D	46
		E41M59_(188C)	3D	188
		E41M59_(147C)	6A	147
		E41M59_272	6A	272
		E41M59_276	6B	276
		E41M59_77	6B	77
		E41M59_(103C)	6B	103
		E41M59_(97CM)	6D	97
		E41M59_47	7B	47
		E41M59_134	unlinked	134
E41M62	6	E41M62_292	3A	292
		E41M62_(114C)	3B	114
		E41M62_(124C)	4A	124
		E41M62_(242C)	4A	242
		E41M62_296	5B	296
		E41M62_(337C)	1A	337

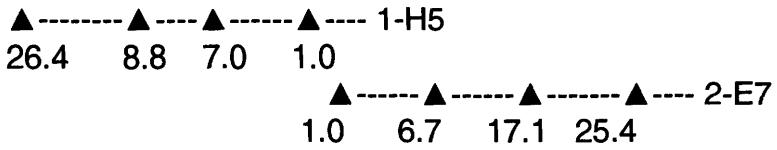
Appendix E Master plate and address of putative CBF clones

Clone	*Plate*	*Address*	*Clone*	*Plate*	*Address*	*Clone*	*Plate*	*Address*
A1_1	11	I20	E8_1	1264	J23	B3_2	2182	M19
A2_1	72	K14	E9_1	1269	D9	B4_2	2193	A9
A4_1	86	G8	E10_1	1291	I2	B5_2	2243	D9
A3_1	97	E20	E11_1	1295	I7	B6_2	2245	A19
A5_1	135	L16	E12_1	1295	M1	B7_2	2245	G9
A6_1	166	P11	F1_1	1297	K11	B8_2	2245	E2
A7_1	172	I19	F2_1	1316	B14	B9_2	2248	A21
A8_1	185	K3	F3_1	1324	J4	B10_2	2257	B5
A9_1	190	H5	F4_1	1351	D20	B11_2	2260	I4
A10_1	206	C13	F5_1	1374	F6	B12_2	2262	L1
A11_1	210	D22	F6_1	1438	F22	C1_2	2281	M20
A12_1	341	J20	F7_1	1449	F6	C2_2	2298	F4
B1_1	347	M11	F8_1	1449	G6	C3_2	2301	E9
B2_1	348	M2	F9_1	1453	H7	C4_2	2351	F19
B3_1	352	N18	F10_1	1455	M12	C5_2	2353	E14
B4_1	354	L3	F11_1	1471	F5	C6_2	2360	L17
B5_1	371	N20	F12_1	1483	O15	C7_2	2370	H20
B6_1	371	H18	G1_1	1516	G4	C10_2	2439	H3
B7_1	384	E5	G2_1	1523	G18	C8_2	2441	B20
B8_1	385	O15	G3_1	1528	P5	C9_2	2500	L20
B9_1	390	M15	G4_1	1548	G4	C11_2	2518	D2
B10_1	400	L3	G5_1	1558	M10	C12_2	2523	K2
B11_1	415	A22	G6_1	1565	F16	D1_2	2523	E6
B12_1	423	P7	G7_1	1583	N10	D2_2	2529	H1
C1_1	432	F20	G8_1	1610	P11	D3_2	2563	M11
C2_1	435	E17	G9_1	1644	N16	D4_2	2568	O12
C3_1	440	E9	G10_1	1685	J24	D5_2	2576	D14
C4_1	452	O11	G11_1	1694	H14	D6_2	2578	G13
C5_1	456	H3	G12_1	1716	E15	D7_2	2667	K5
C6_1	461	B19	H1_1	1740	J17	D8_2	2671	E8
C7_1	463	B18	H2_1	1772	O2	D9_2	2674	J8
C8_1	485	G3	H3_1	1791	J12	D10_2	2689	C5
C9_1	492	J10	H4_1	1811	N7	D11_2	2789	E10
C10_1	494	G8	H5_1	1811	M14	D12_2	2818	G11
C11_1	625	M6	H6_1	1847	M8	E1_2	2838	D22
C12_1	626	L8	H7_1	1863	N13	E2_2	2869	E23
D1_1	645	G21	H8_1	1904	G16	E3_2	2874	G4
D2_1	652	B21	H9_1	1957	O17	E4_2	2892	G17
D3_1	655	A22	H10_1	1965	C6	E5_2	2924	H10
D4_1	671	G19	H11_1	1977	G4	E6_2	2986	E14
D5_1	678	N9	H12_1	1978	G8	E7_2	3005	K17

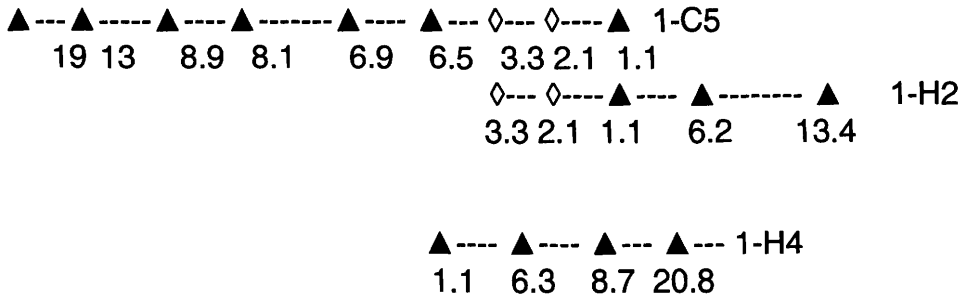
D6_1	679	K23	A1_2	1980	B7	E8_2	3007	K10
D7_1	697	A22	A2_2	1981	H3	E9_2	3051	A18
D8_1	757	J21	A3_2	1986	B20	E10_2	3065	A23
D9_1	760	K11	A4_2	2014	H1	E11_2	3077	N18
D10_1	783	B10	A5_2	2022	L16	E12_2	3175	O8
D11_1	926	H9	A6_2	2029	K22	F1_2	3208	C10
D12_1	1065	H19	A7_2	2070	F8	F2_2	3223	C5
E1_1	1100	N2	A8_2	2079	D22	F3_2	3223	G8
E2_1	1111	G23	A9_2	2098	F7	F4_2	3232	K10
E3_1	1162	I12	A10_2	2128	C7	F5_2	3249	C12
E4_1	1179	D19	A11_2	2144	H6	F6_2	3259	O12
E5_1	1236	P23	A12_2	2148	N17	F7_2	3296	C21
E6_1	1259	O9	B1_2	2166	F16			
E7_1	1261	A15	B2_2	2170	E6			

Appendix F Groupings of putative CBF clones. The 16 groupings are based on fragments from *Hin*dIII restriction digestion with similar CBF hybridizing bands (▲) and similar non-CBF bands (◊) after probing with a ^{32}P-labelled CBF probe.

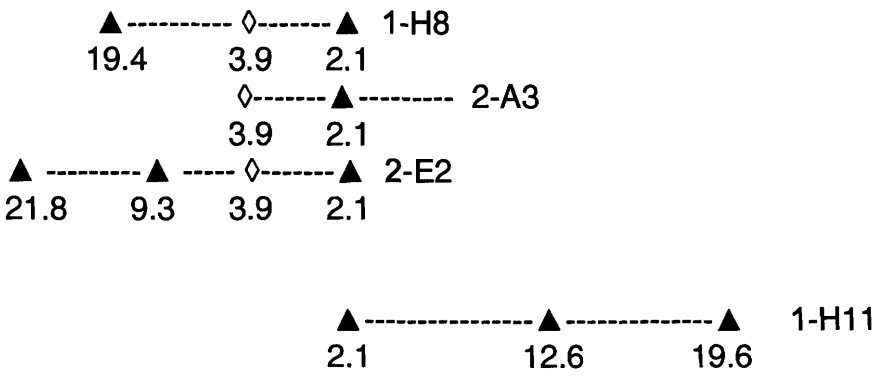
Group 1-clones with similar 1.0 kb CBF hybridizing band



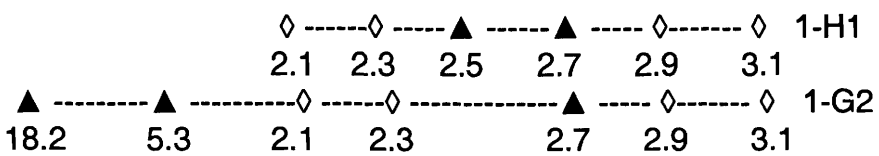
Group 2-clones with similar 1.1 kb CBF hybridizing band



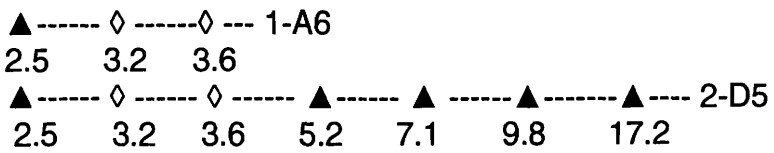
Group 3-clones with similar 2.1 kb CBF hybridizing band



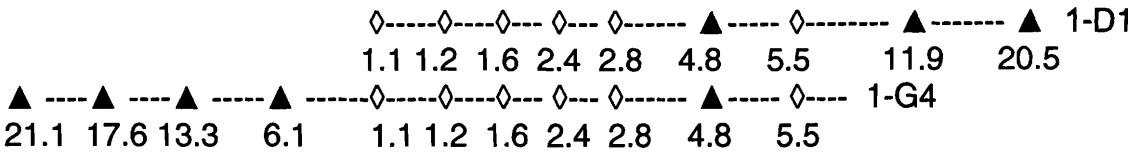
Group 4-clones with similar 2.7 kb CBF hybridizing band



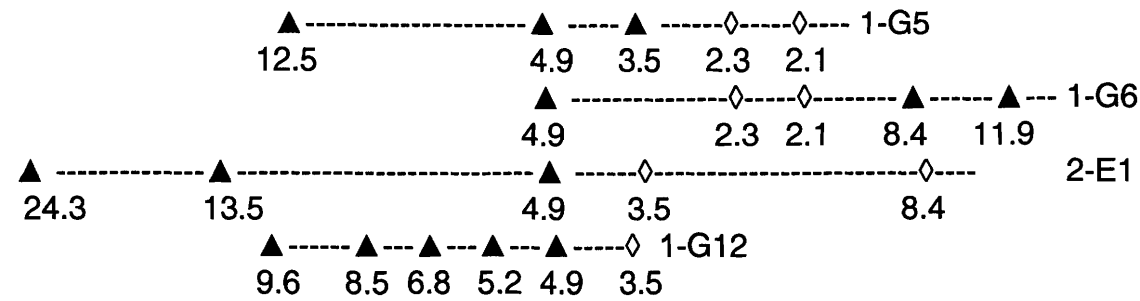
Group 5-clones with similar 2.5 kb CBF hybridizing band



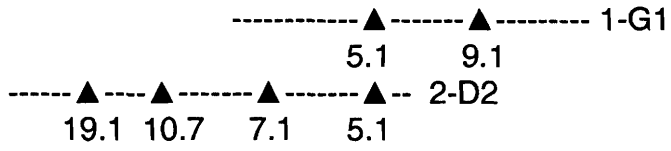
Group 6-clones with similar 4.8 kb CBF hybridizing band



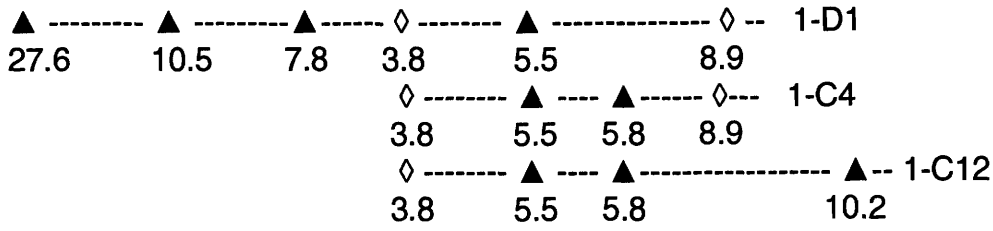
Group 7-clones with similar 4.9 kb CBF hybridizing band



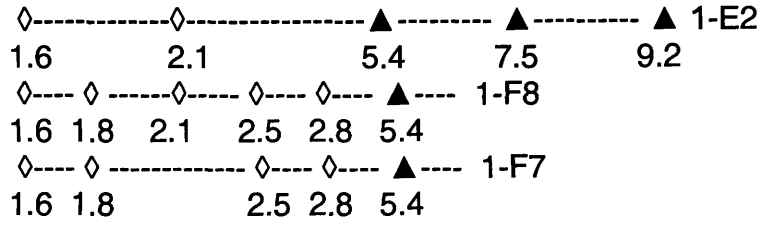
Group 8-clones with similar 5.1 kb CBF hybridizing band



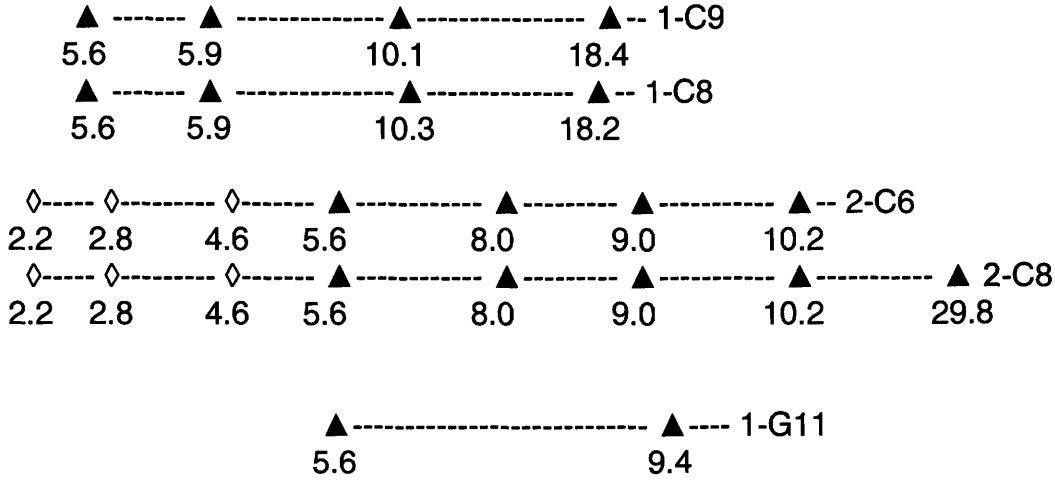
Group 9-clones with similar 5.5 kb CBF hybridizing band



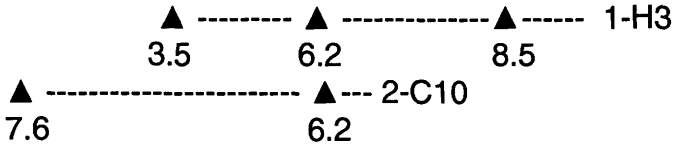
Group 10-clones with similar 5.4 kb CBF hybridizing band



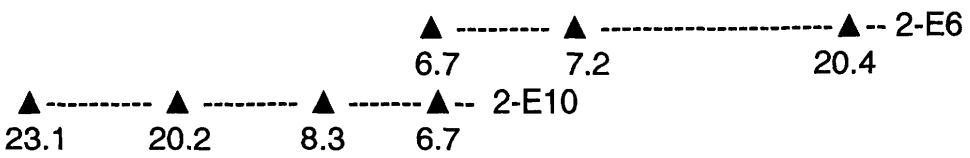
Group 11-clones with similar 5.6 kb CBF hybridizing band



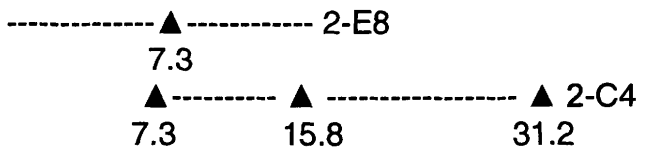
Group 12-clones with similar 6.2 kb CBF hybridizing band



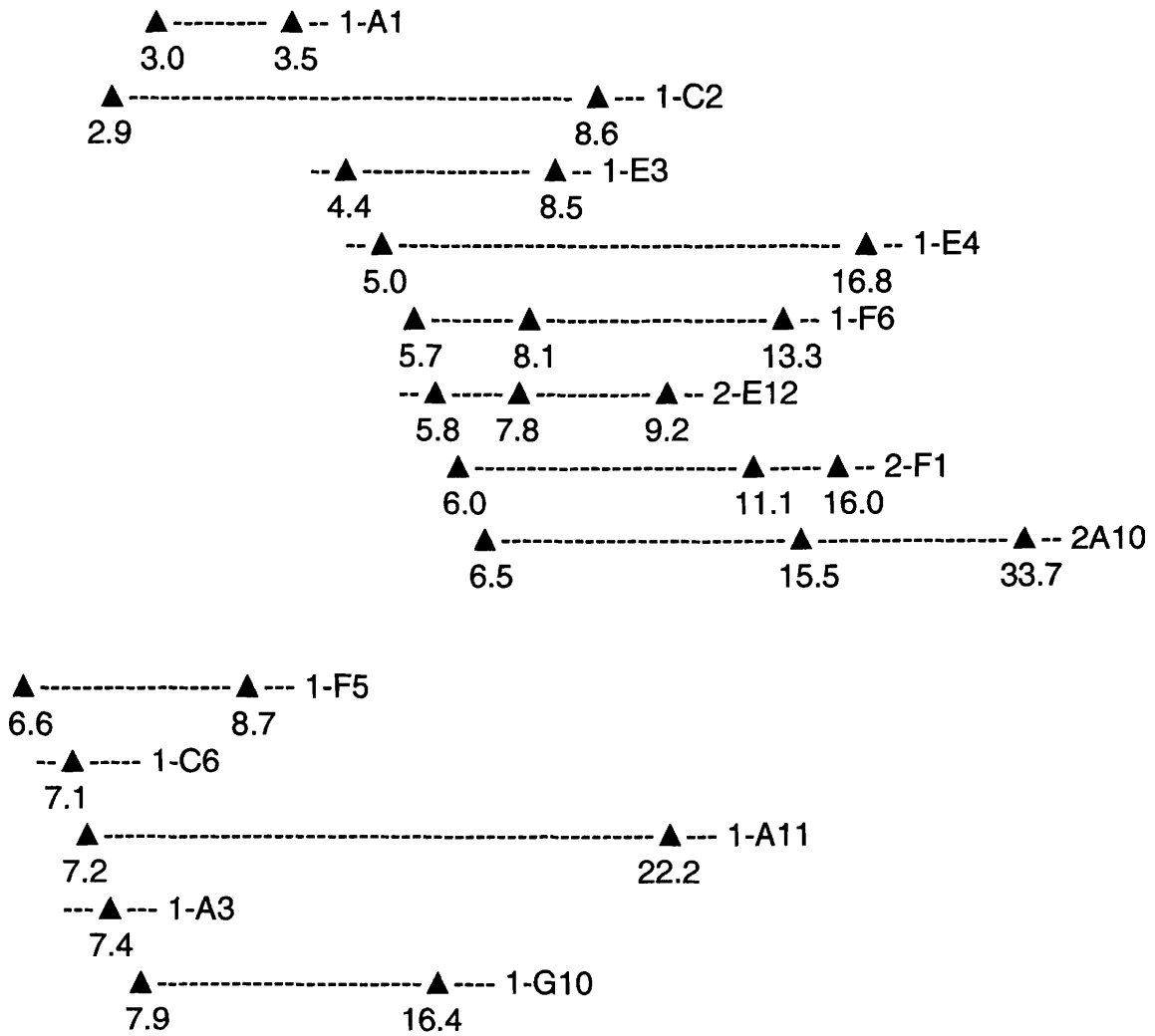
Group 13-clones with similar 6.7 kb CBF hybridizing band



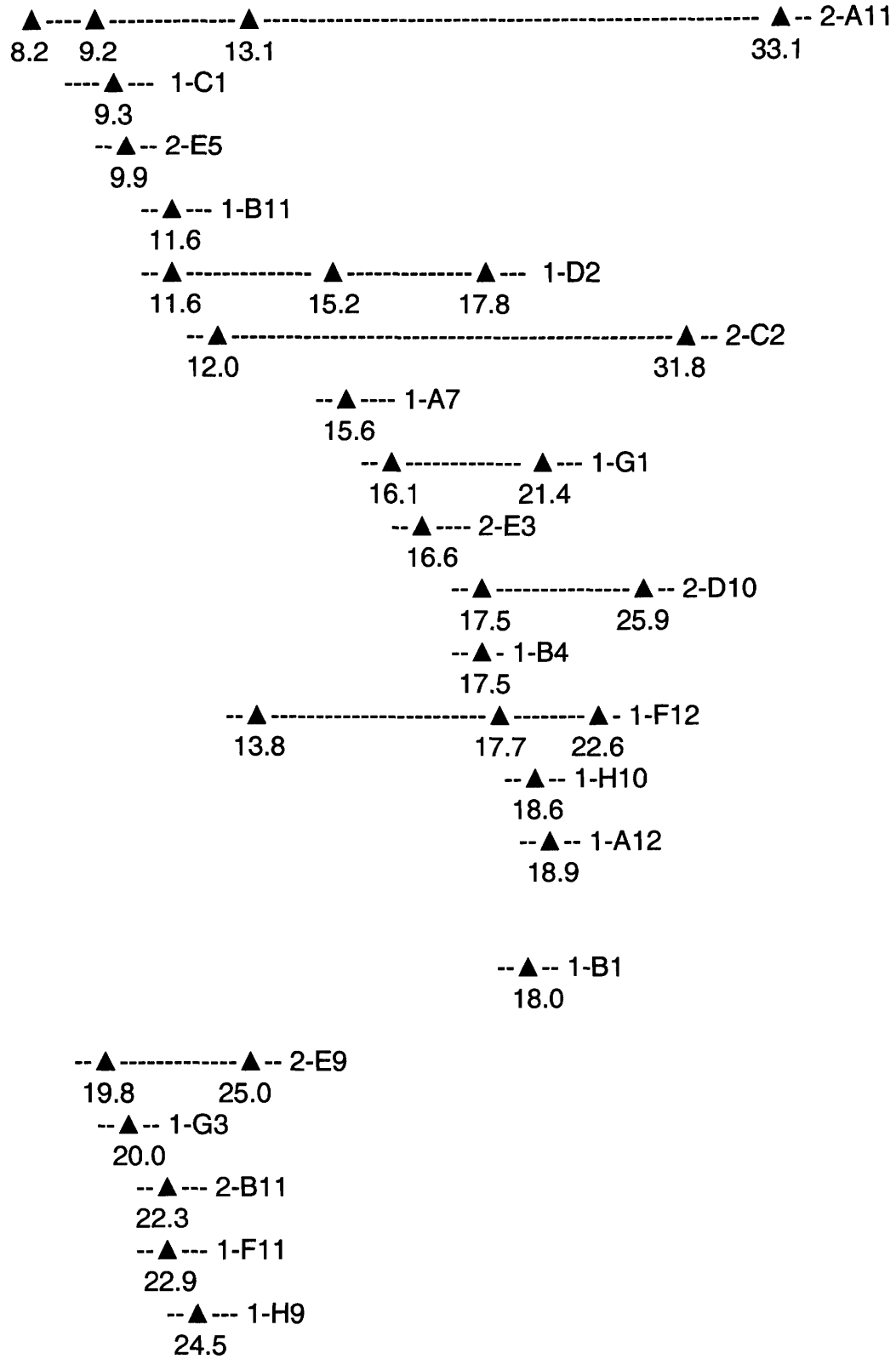
Group 14-clones with similar 7.3 kb CBF hybridizing band



Group 15-clones with unique CBF hybridizing band



Group 16-clones with CBF hybridizing band > 8.0 KB



Appendix G Cultivar X media interaction. Means with the same letter are not significantly different based on LSD test ($P \leq 0.05$). Media which were the most responsive for a given cultivar are indicated by an asterisk (*).

Cultivar	*Media*	*Mean no of shoots/media*
Osprey	T2B1	360.21 a*
	T1B1	336.27 a*
	T2B2	334.47 a*
	T1B2	296.33 b
	MBT1	221.30 c
	MBT2	207.21c
	MBR4A	40.40 d
	MBR3A	30.79 d
Clair	T2B1	366.73 a*
	T2B2	349.53 a*
	T1B1	304.00 b
	T1B2	303.23 b
	MBT1	202.56 c
	MBT2	134.65 d
	MBR4A	37.20 e
	MBR3A	30.82 e
Ptarmigan	T2B1	425.20 a*
	T2B2	419.53 a*
	MBT2	401.8 ab
	T1B1	364.93 bc
	T1B2	350.00 c
	MBT1	327.87 c
	MBR4A	44.53 d
	MBR3A	42.33 d
Nanda	T2B1	478.06 a*
	T2B2	425.20 b
	T1B2	400.06 b
	T1B1	389.80 b
	MBT2	266.60 c
	MBT1	226.90 c
	MBR4A	48.67 d

	MBR3A	44.64 d
Plenty	T1B1	580.53 a*
	T1B2	539.47 a*
	T2B2	453.13 b
	T2B1	440.73 b
	MBT2	403.55 b
	MBT1	332.95 c
	MBR4A	90.33 c
	MBR3A	43.40 d